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Molecularly imprinted polymers for separation of various sugars from human urine

Burcu Okutucu*, Seçil Önal

Ege University, Faculty of Science, Department of Biochemistry, 35100 Bornova-Izmir, Turkey

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ABSTRACT

Molecularly imprinted polymers were the new, simple and unexpensive materials that can be used in several clinical applications. Phenylboronic acid has been frequently used as functional monomer for the covalent imprinting of diols. In this study, the phenylboronic acid esters of fructose, galactose, glucose and raffinose were synthesized and then used as template analytes. The adsorption capacities of fructose, galactose and glucose–phenylboronic acid imprinted polymers were 75, 10 and 30%, respectively. The batch rebinding studies and Scatchard analysis were done for all sugar imprinted polymer. Glucose is one of the mostly found sugar in the urine. The glucose:phenylboronic acid imprinted polymer was used for the analysis of glucose, fructose, galactose, sucrose, maltose, lactose and raffinose in spiked urine. The selectivity of glucose:phenylboronic acid imprinted polymer to urine monosaccharides was found as nearly 45–55% and to di- and polysaccharides was found as 30–35%, respectively.

1. Introduction

Molecular recognition is the basis of many biological processes associated in maintaining life. Molecular imprinting is an attractive technique for the synthesis of highly selective polymeric receptors having artificial generated recognition sites. These materials are synthesized with polymerizable functional monomers and crosslinkers that are surrounded around a template molecule. After polymerization, template molecules are removed leaving in the polymer selective recognition sites with shape, size and functionalities complementary to the template [1–6]. Functional monomers are arranged around the template by a covalent or non-covalent bonding procedure. In covalent approach, the template is chemically bound to a polymerizable monomer via a labile covalent bond. This bond is then cleaved to remove the template molecule, leaving binding cavities that are claimed to be more uniform in placement of a single complementary functional group than those obtained by the non-covalent approach. In non-covalent approach, polymerizable functional monomers mixed with a template in the presence of a large portion of crosslinker. After the pre-arrangement between functional monomers and template (electrostatic interaction, hydrogen bonding or similar non-covalent bonds), the template-functional monomers and crosslinker are copolymerized [7–10].

The molecular recognition of carbohydrates becomes remarkable due to their importance as biomarkers in different biological systems. Carbohydrates play an important role in processes as mediation of cell adhesion, intracellular recognition, fertilization and as targets of bacterial/viral infection of cells [11]. There are various techniques for the estimation of total carbohydrate concentration, such as electrochemistry, near infrared spectroscopy, optical rotation, colorimetry and chromatography. Chromatographic techniques, such as thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) are commonly used to separate and identify carbohydrates. Carbohydrates can be separated on the basis of their adsorption coefficients, polarities and/or sizes with these chromatographic techniques. HPLC is currently the most important chromatographic method for analyzing carbohydrates because it is capable of rapid, specific, sensitive and precise measurements. However these chromatographic methods suffer from many drawbacks, such as GC requires that the samples must be volatile, to be derivatized and HPLC samples can often be analyzed with specific reagents. Carbohydrates can also be separated by electrophoresis after they have been derivatized to make them electrically charged. These are all time consuming and also expensive techniques.

Molecularly imprinted polymers (MIP) offer the possibility to distinguish according to spatial arrangement of functional groups to provide specific interactions with the template when rebinding. Also resulted molecularly imprinted polymers are stable, roboust and resistant to a wide range of pH and solvents. For all of these reasons, MIPs have been used in the analysis of carbohydrates as

* Corresponding author. Tel.: +90 232 3438624; fax: +90 232 3115485.
E-mail address: burcu.okutucu@ege.edu.tr (B. Okutucu).

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chromatographic resins, sensors and solid phase extraction adsorbents [12–16]. Some mono- and disaccharides have been imprinted by the use of covalent or non-covalent bonding between monomer and template [17–20].

Boronic acids have extensively been used to design monomeric or polymeric tailor-made synthetic receptors for sugar recognition due to their tendency to form reversible boronate esters with cis-diols of carbohydrates. A large variety of MIPs were prepared via covalent approach by dehydrocondensation reaction with polymerisable boronic acid derivates and carbohydrates. This broad interest in boronic acids results from their relatively low toxicity and the unique interactions they have with diols, namely they form cyclic esters with diols in water much more readily than many other acids [21,22].

In this study, we aimed to synthesize the covalently imprinted sugar–phenylboronic acid polymers and also to determine the sugar recognition abilities of the polymers in urine. The esters of fructose, galactose, glucose and raffinose were synthesized and used as template analytes. Appropriate ratio of crosslinking agent, initiator, porogen and template were mixed. Thermal polymerization was used for the preparation of sugar imprinted polymers. The templates were then removed by different hydrolytic solutions and the best condition was determined for each imprinted polymer. The binding characteristics of MIPs and templates were evaluated using batch rebinding experiments. Scatchard plot analysis was also investigated. The selectivity of each polymer to different saccharides (fructose, glucose, galactose, sucrose and raffinose) was also determined in protic solvent and urine.

2. Experimental

2.1. Materials

Glucose (Glc), galactose (Gal), fructose (Fru), raffinose (Raf), sucrose (Suc), phenylboronic acid (PBA), H_2SO_4, chloroform (CHL), methanol (MeOH), acetic acid (AcA) and ethyleneglycol dimethacrylate (EGDMA) were obtained from Sigma Chem. Co. (St. Louis, USA) and azobisisobutyronitrile (AIBN) was purchased from Wako Pure Chem. Ind. (Osaka, Japan). The EGDMA and AIBN were reagent grade and were used without any further purification. Dimethyl formamide (DMF) was obtained from Carlo Erba (Italy). All other chemicals and reagents were of the highest available purity and used as purchased.

2.2. Esterification of sugars with phenylboronic acid

The phenylboronic acid ester derivatives of sugars (galactose, glucose, fructose, sucrose, raffinose) were synthesized according to Wulff et al. [9]. The ratios of sugar:phenylboronic acid (w/w) were 1:2 for Glc:PBA, Gal:PBA, Suc:PBA and Raf:PBA and 1:1 for Fru:PBA. The appropriate amount of sugar and phenylboronic acid was esterified in 30 ml of DMF (H_2SO_4 was used as a catalyst) by azeotropic distillation (80 °C). After 2 h, the reaction mixture was centrifuged at 10,000 rpm for 30 min (Hettich Universal 30 RF). The precipitate was washed by methanol and then evaporated to dryness [2].

2.3. Preparation of the sugar–PBA imprinted polymer

10 mg of sugar–phenylboronate esters was dissolved in 5 ml of chloroform and then 20 mmol of ethyleneglycol dimethacrylate was added as a crosslinker. The reaction mixture was sonicated (Ultrasonic LC 30) for 30 min and degassed 5 min with $N_{2(g)}$. The polymerization was initiated by addition of azobisisobutyronitrile and carried out at 60 °C for 12 h. A control polymer was prepared with phenylboronic acid instead of sugar esters (control polymer; non-imprinted polymer). The polymers were dried in vacuum at 60 °C for 12 h and then ground to particles of 50 μm diameter or smaller (Retsch RM 100). Galactose, glucose and fructose phenylboronate esters were removed by methanol:water (1:1) for three rounds and raffinose phenylboronate ester was removed by methanol:conc. HCl (1%). The amounts of galactose, glucose and fructose extracted from MIPs were determined by the 3,5-dinitrosalicylic acid (DNS) assay [23] and raffinose was assayed with o-cresol:sulphuric acid reagent [24]. To obtain a more rational design for MIP synthesis, a rapid tool to evaluate the interaction between the monomer and template in solution as a mimic for pre-polymerization was employed by spectroscopic method. The spectral profiles of sugars, phenylboronic acid and sugar–phenylboronic acid esters were measured in chloroform [25].

2.4. Batch rebinding assay

The sugar binding efficiencies of the imprinted polymer were studied by batch rebinding assay. Rebinding experiments were studied with the imprinted polymers and control polymer by using different concentrations of sugars (0.1–9 μmol/ml). Briefly, 5 mg of the each polymer (Gal:PBA, Glc:PBA, Fru:PBA and Raf:PBA imprinted polymer) and control polymer were mixed with sugars in 1 ml of MeOH:water (1%) and then incubated on rotator (Stuart rotator SB3) at room temperature for 4 h at 25 rpm/min. After that, the polymers were centrifuged at 10,000 rpm for 10 min. The remaining substrate concentrations were determined by DNS assay and with o-cresol:sulphuric acid reagent. All the rebinding experiments were run in triplicate and the difference in the readings in triplicates was less than ±5%. The control polymer was used to determine the non-specific binding. The amount of sugars that bound to the polymers (Bound %) was defined according to the equation;

$$\text{Bound}\,\% = \frac{C_o - C_s}{C_s} \times 100$$

where C_o is the initial concentration of sugar and C_s is the concentration of sugar in the supernatant after rebinding.

In order to determine the effect of pH on the rebinding, different buffers with various pH values: 0.1 M acetate (pH 4–5), phosphate (pH 6–7–8) and glycine:NaOH (pH 9–10) were used.

2.5. Selectivity of sugar boronate ester imprinted polymers toward different sugar

The substrate selectivities of sugar boronate ester imprinted and control polymers were determined with different concentrations of galactose, glucose, fructose, sucrose and raffinose in equal amounts. 10 mg of polymer and suitable amounts of sugars were incubated in 1 ml of MeOH:water (1%) by rotator (25 rpm/min) for 4 h at room temperature. After that, the mixture was centrifuged at 10,000 rpm for 10 min and the concentrations of the remaining sugars were measured.

2.6. Extraction of various sugars from spiked human urine with Glc:PBA imprinted polymer

Urine was collected from healthy person and then immediately centrifuged at 3000 rpm for 30 min. The supernatant was collected and then diluted (100 fold) with water without any other pretreatment. To evaluate the feasibility of the imprinted polymer for real biological sample, 30 mg of Glc:PBA imprinted polymer was immerged in spiked urine which is containing each of the different concentration (50–250 μg/ml) of sugars (fructose, galactose, sucrose, maltose, lactose and raffinose). The sugar loaded polymers were washed with MeOH:water (1:1) and washings were

analyzed for the amount of sugar. The normal urine was also tested by DNS assay and no meaningful result was obtained (no sugar was detectable).

3. Results and discussion

3.1. Synthesis of the sugar:PBA imprinted polymers

The boronic acid template molecule has been used widely for the synthesis of sugar specific imprinted polymers considering the affinity of boronic acid moieties toward diols [12]. We preferred to synthesize the PBA imprinted polymer by covalent imprinting. The covalent approach was chosen to control the stoichiometry of functional monomer to template molecule. By covalent interactions certain functionalities can be introduced in a defined number and orientation into the active site of polymer. There is also no need to use excess monomer concentration in covalent imprinting. In non-covalent imprinting, while using excess amount of monomer was resulted heterogeneous recognition sites, more non-specific binding and pre-polymerization complex were not stable enough for further steps. The main important step before the synthesis of sugar–phenylboronic acid imprinted polymer was the esterification. The ester bond between sugar and phenylboronic acid was necessary to keep the template stable for precise imprinting. Boronic acids which aim to produce an exact fit by reforming the stable bonds is one of the monomer that have been used in covalent imprinting. We have succeeded in the synthesis of different sugar–phenylboronic acid esters except sucrose ester. Because of the anomeric hydroxyl of glucose which is linked to fructofuranose at the 2′ position, sucrose does not form a complex with boronic acid [25,26]. As it is expected, we did not get any precipitate after esterification reaction of sucrose with phenylboronic acid. The sucrose recognition ability of the other sugar:phenylboronic acid imprinted polymers were also studied.

The polymer cavity thus formed contains the boronic acid residues in the exact spatial arrangement necessary to rebind the sugars. Generally the removal of the template from covalently imprinted polymer requires only a mild hydrolysis such as aqueous methanol. As ester hydrolysis involves the addition of one mole of water to each of the cleavage points, steric and electrostatic repulsions were increased. In order to remove the sugar:PBA ester from the imprinted polymer, we have examined different solutions; MeOH, MeOH:water (1:1; v/v), MeOH:1 M NaOH (1:1; v/v), MeOH:AcA (4:1; v/v) with several times (Fig. 1). All the washing experiments were run in triplicate and the difference in the readings in triplicates was less than ±5%. The best results were obtained with MeOH:water (1:1; v/v) and nearly 75% of templates were removed. Because of the complex structure and the undefined ester chemistry of raffinose, some problems have been appeared while removing from the polymer. As it is known, the stability of carbohydrate complex with borate and phenylboronic acid is often determined by the presence of free glycosidic hydroxyl groups. The tautomeric form of the sugar in aqueous solutions is one of the key factors regarding binding strength [27]. To be an example, the boronate complexes of lactose are weaker than those of its two constituents [28]. Removing of raffinose from the polymer was studied by different washing solutions mentioned above. These solutions could not be able to remove the raffinose from the polymer. Instead of them MeOH:conc. HCl (1%) (1:1; v/v) was studied and chosen as the best for the removing of raffinose (nearly 50%) (Fig. 1).

3.2. Batch rebinding studies

Adsorption capacity is an important index to determine the adsorption properties of adsorbents. It determines how much adsorbent is required to quantitatively concentrate the analytes from a given solution. Some previous works have shown that, MIPs often exhibit higher imprinting effect in the solvent which is used as the porogen in the polymerization [29]. In our study, we chosen MeOH instead of chloroform (porogen) as a rebinding solvent for being a polar protonic solvent. The adsorption of MIP/NIP is synchronously suppressed with methanol and this resulted in both the specific and non-specific adsorptions being much less in MeOH than chloroform. The adsorption capacities of sugar–PBA ester imprinted polymer and control polymer were shown in Fig. 2. As is seen from the Fig. 2, the galactose (Fig. 2a) and glucose–PBA (Fig. 2b) imprinted polymers have very less adsorption capacities than fructose–PBA (Fig. 2c) imprinted polymer. This is an expected result because of the shape and orientation of the functional group inside the cavities. Ayyub et al. [26] and Smoum and Srebnik [21] mentioned that boronic acid can bind to both 1,2 and 1,3 cis diols but preferentially binds to 1,2 cis diols. As is known, glucose does not have a 1,2 cis diol but has a 1,3 cis diol in its dominate pyranose form and it binds to boronic acid in its furanose form.

The interesting point was the adsorption graph of raffinose–PBA imprinted polymer (Fig. 2d). Raffinose is a trisaccharide that contains galactose, glucose and fructose. The binding competition of sugars to boronic acid is generally depending on the relative concentration of sugar and boronic acid. We have used in equimolar of galactose, glucose, fructose and raffinose. The binding amount increases gradually with the increase of the concentration of raffinose and it could not reach a stable value because in the case of raffinose polymer the amount of galactose, glucose and fructose is higher than the Gal:PBA, Glc:PBA and Fru:PBA. Therefore there is no any spatial arrangement for the binding of any other galactose, glucose and fructose.

Batch rebinding experiments were performed in MeOH:water (1%; v/v) with different pH values for the determination of pH effect on rebinding assays. Buffer does play an important role in influencing the binding constants of boronic acid and diol. Boronic acid is a Lewis acid. The presence and concentration of Lewis bases (phosphate, chloride, tris, vs.) are expected to affect the complexation state of the boron atom. One of them is the phosphate buffer effect. The ionization state of phosphate ion was changed at different pH values. The other reason may be the conformational changes and sterics interactions which are all idiosyncratic factors associated with each specific binding equilibrium that can affect the binding. Another point is the effect of solvent on diol complexation. The addition of MeOH to an aqueous solution increased the rebinding [30]. The best rebinding ratios were obtained at pH 8.0 for galactose and raffinose, and at pH 7.0 for other sugars with phosphate buffer.

A binding isotherm measures binding efficiency of a polymer over a range of analyte concentrations and is usually plotted as the concentration of analyte bound to a polymer (B) versus the concentration of free analyte remaining in solution (F). Binding isotherms for MIPs can be obtained from batch rebinding studies in which a constant weight of a polymer is equilibrated with a known concentration of analyte [31,32]. The quantity of binding sites of the MIPs was examined by using Scatchard analysis. Scatchard equation is as follows;

$$\frac{Q}{[C]} = \frac{(Q_{max} - Q)}{K_D}$$

where Q is the amount of template bound to the polymer, Q_{max} is the apparent maximum number of binding sites, K_D is the equilibrium dissociation constant and $[C]$ is the equilibrium constant of template. By constructing Scatchard plots (B/F vs. B) for sugar:PBA imprinted polymers (galactose:PBA (Fig. 3a), glucose:PBA (Fig. 3b), fructose:PBA (Fig. 3c) except raffinose:PBA (Fig. 3d)) show a linear relationship. The Scatchard plot for raffinose:PBA imprinted polymer was curved. This curvature has been an evidence for binding

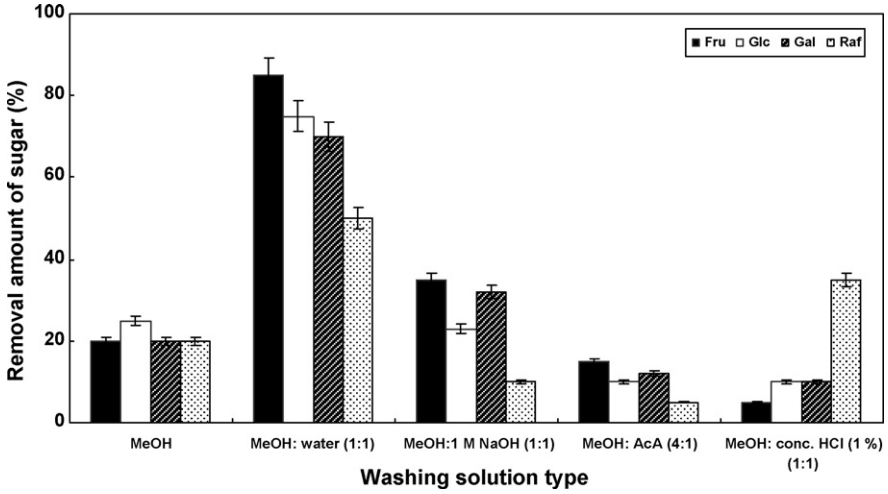

Fig. 1. Removing amount of sugars from the imprinted polymers (polymer amount: 100 mg; volume: 3 ml).

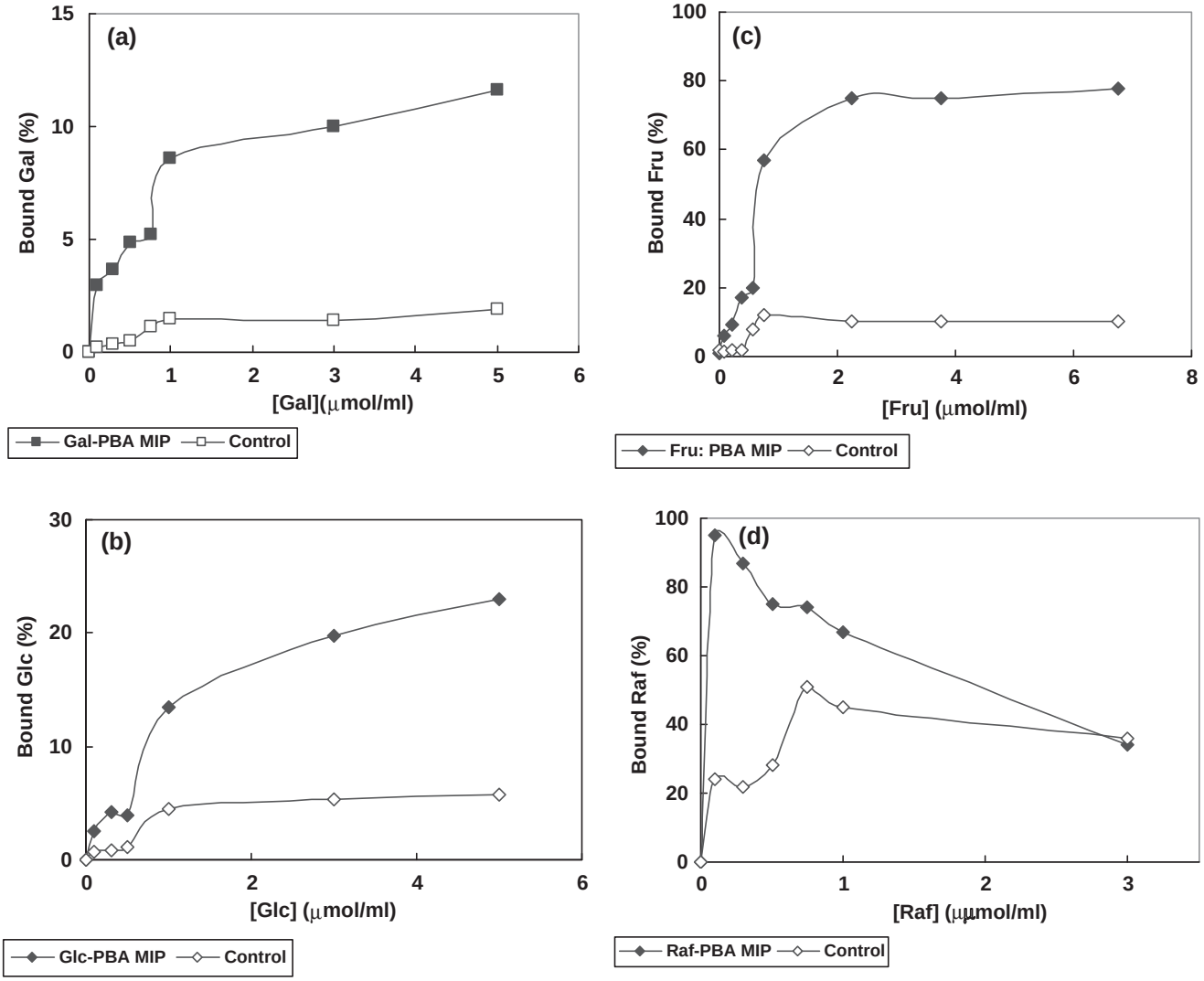

Fig. 2. The adsorption capacities of galactose:PBA (a), glucose:PBA (b), fructose:PBA (c) and raffinose:PBA (d) imprinted polymer.

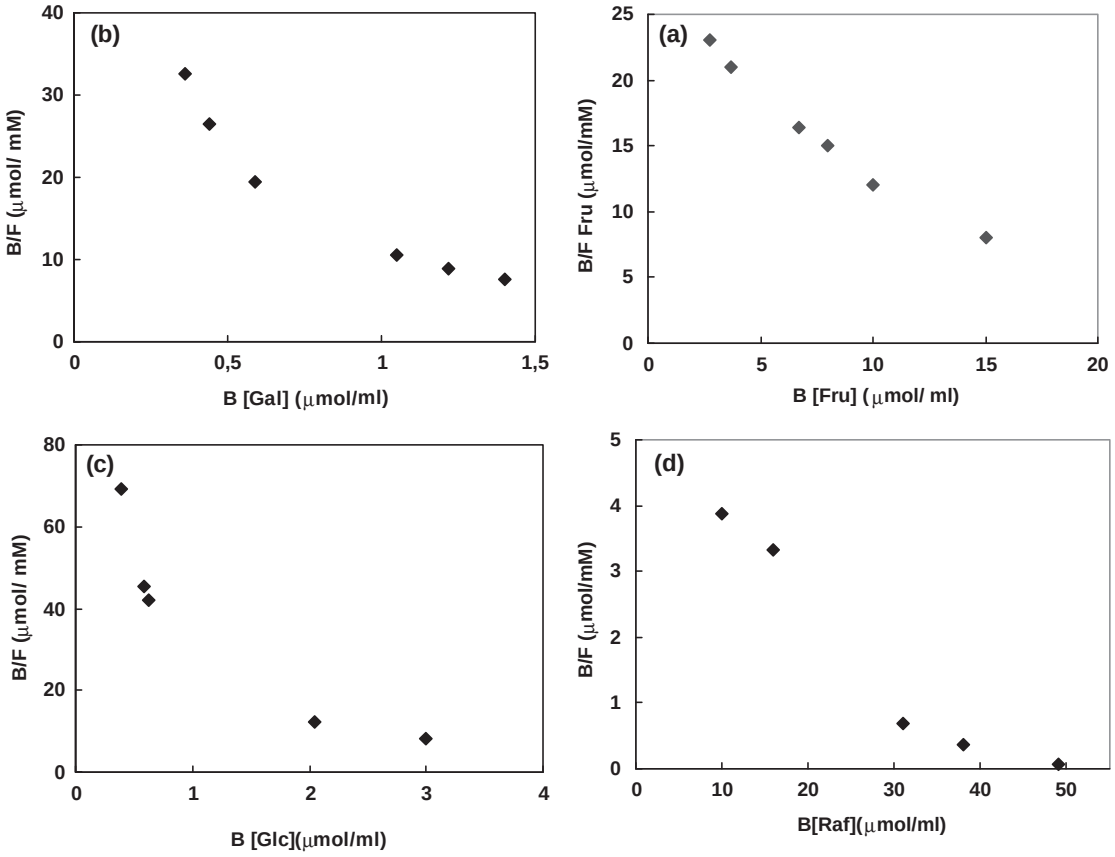

Fig. 3. The Scatchard graphs of galactose:PBA (a), glucose:PBA (b), fructose:PBA (c), raffinose:PBA (d) imprinted polymers.

site heterogeneity. The distribution of these heterogeneous binding sites is the result of the amorphous nature of the polymer matrix, stepwise complexation between the template and functional monomers and incomplete removal of the template from the MIP. The K_D values of sugars were given at Table 1.

3.3. Selectivity studies

The ability of discrimination of each sugar:phenylboronic acid ester imprinted polymer was evaluated by template and structural analogue. The distribution coefficient (k) was utilized to evaluate the molecular selectivity of polymers. k is defined as follows:

$$k = \frac{C_p}{C_s}$$

where C_p (μmol g^{-1}) is the amount of sugars adsorbed on polymers and C_s (μmol/ml) is the equilibrium concentration of sugars in solution.

The sugar:PBA imprinted polymers have different k values (Table 2). As is also mentioned above the raffinose:PBA imprinted polymer has much more efficiency to galactose than other sugars. The similar k values of galactose and glucose were the reason of diol arrangement of them with boronic acid.

Table 1
The K_D values of sugar–phenylboronic acid imprinted polymer.

	MIP	NIP
Fru–PBA	0.8	0.004
Gal–PBA	0.65	0.006
Glc–PBA	0.7	0.003
Raf–PBA	0.15	0.005

3.4. The recognition ability of Glc:PBA imprinted polymer from sugar spiked urine

Urine is one of the most extensively analyzed biological fluids in clinical investigations owing to its high availability and non-invasive collection. Nevertheless, to determine any biomolecule in urine samples need pretreatment techniques because of low concentration of target analytes, inherent complexity of the matrices (urine contains nonprotein nitrogen metabolites, carbohydrates and proteins, in concentrations of less than 0.6–10 mg/ml, 44–500 μg/ml and 0.1–20 μg/ml, respectively) and even the incompatibility with the instrumentation, which limits the sensitivity and selectivity of the measurement [33]. Several metabolic disorders are characterized by the excretion of abnormally large quantities of sugar(s) in the urine. Certain inborn errors of metabolism and diabetes mellitus were the most known diseases. The carbohydrates mostly found in urine were D-glucose, D-galactose, D-fructose, D-mannose, sucrose, D-ribose, lactose, maltose, L-arabinose, and D-xylose [34,35]. The techniques that have been employed for the analysis of urine carbohydrates were paper, thin layer, high performance liquid chromatography and capillary electrophoresis. The disadvantage of paper and thin layer chromatography were the resolution and sensitivity. They are lack of resolution of enantiomers and need too much urine sample [36]. Nowadays, clinical applications of MIPs are very attractive area [37–39].

Human urine was spiked with three different concentrations of sugars (50–250 μg/ml) and the recoveries (%) are given in Table 3. Different dilutions (1:5, 1:25 and 1:100) of urine were also studied; because of too much matrix effect, there is much interference in sugar analysis. 1:100 dilution of urine was used for the best reality results. As is shown in Table 3, for monosaccharides at lower sugar concentrations, the recoveries of spiked urine were expectable.

Table 2
The distribution coefficient of polymers with different sugars.

MIP[a]	k (μmol/mM)				
	Fru	Gal	Glc	Suc	Raf
Fru–PBA	–	0.082	0.2	0.004	0.06
Gal–PBA	0.12	–	0.28	0.006	0.09
Glc–PBA	0.15	0.25	–	0.004	0.07
Raf–PBA	0.075	0.09	0.009	0.002	–

[a] Polymer amount: 10 mg; binding time: 4 h, initial concentration of sugars: 5 μmol/ml; volume: 1 ml; solvent: MeOH:water (1%).

Table 3
The recovery of monosaccharide spiked urine.

Sugar	Spiked (μg/ml)	Found (μg/ml)	Recovery (%)
Glucose	50	24	48
	100	66	66
	250	138	55
Galactose	50	20	40
	100	47	47
	250	97.5	39
Fructose	50	21.5	43
	100	55	55
	250	138	55
Lactose	50	22	44
	100	35	35
	250	85	34
Maltose	50	18	36
	100	25	25
	250	60	24
Sucrose	50	15	30
	100	30	30
	250	35	14
Raffinose	50	20	40
	100	32	32
	250	55	22

At high concentrations like, 100 or 250 μg/ml the recovery values were near the same. The reason is that most of the sites have been fulled with exact fit. The recovery values of di- and polysaccharides were less than monosaccharides (Table 3). It can be attributed by the complexity of sugars that affect the sugar–diol complex formation.

4. Conclusion

Boronic acid containing polymers have found utility in a variety of biomedical applications [40]. One of them is for recognition of sugars effectively. The sugar (galactose, glucose, fructose, raffinose)–PBA esters were synthesized by esterification and then used as templates in covalently imprinted polymer. The sugar:phenylboronic acid ester imprinted polymers can be a good candidate for identification of sugars (galactose, glucose, fructose, raffinose) separately or together. Boronic acid imprinted polymers will be valuable as selective chromatographic stationary phase for various clinical applications. The synthesizing of molecularly imprinted polymer needs fewer reagents than other chromatographic techniques that were used in analyzing of sugars in urine. The synthesized Glc:PBA imprinted polymer for recognition of sugars from urine was also shed light on its suitability for separation of sugars in different medical areas.

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