Research Article

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Development of molecularly imprinted Acrylamide-Acrylamido phenylboronic acid copolymer microbeads for selective glycosaminoglycan separation in children urine

Çocuk idrarındaki glikozaminoglikan ayrımı için moleküler olarak basılmış Akrilamid-Akrilamido Fenilboronik asit kopolimer mikroboncuklar geliştirilmesi

Abstract

Background: In this study, we synthesized molecularly imprinted copolymers for liquid chromatography columns as a separator for glycosaminoglycan (dermatan sulfate; DS and chondroitin sulfate; CS) in urine.

Materials and methods: Acrylamide and acrylamido phenylboronic acid were used as monomers, acrylamide was used for as base monomer to attract negatively charged groups and acrylamido phenylboronic acid (AAPBA) residues used to form diol bonds between sugar and boronic acid residues to strengthen the attraction. These monomers were synthesized by using precipitation polymerization to form uniform spheres, which are more durable for the pressurized chromatographic systems. Trimethylolpropane trimethacrylate and AIBN were used as crosslinker and starter, respectively.

Results: These GAG selective polymers were filled by pressurized flow into the steel (4.6 mm×1.6 mm) columns, then imprinted GAGs were extracted and analyzed to calculate binding capacity of each milligram polymer. Calibration curves of the GAG selective columns were obtained 62.5–1000 ng/mL less than 5% coefficient variation, and lower matrix effect.

Conclusion: Our imprinted columns separated different GAGs from urine specifically and sensitively. Matrix effect was at an ignorable level thus the challenging use.

Keywords: Glycosaminoglycan; Chromatography; Molecular imprinting; Acrylamido phenylboronic acid; Solid phase extraction.

Öz

Amaç: Bu çalışmada idrarda glikozaminoglikan (Dermatan Sülfat; DS ve Kondroitin Sülfat; CS) ayrılması için sıvı kromatografi kolonları içinde moleküler olarak basılmış kopolimerler sentezlenmiştir.

Gereç ve yöntem: Monomer olarak akrilamid ve akrilamido fenilboronik asit kullanıldı, negatif yüklü grupları çekmek için monomer olarak akrilamid ve şeker gruplarının çekimini güçlendirmek için şeker ve boronik asit kalıntıları arasında diol bağları oluşturmak için kullanılan akrilamido fenilboronik asit (AAPBA) kısımları kullanıldı. Bu monomerler, basınçlı kromatografik sistemler için daha dayanıklı olan muntazam küreler oluşturmak için çökeltme polimerizasyonu kullanılarak sentezlenmiştir. Trimetilolpropan trimetakrilat ve AIBN sırasıyla çapraz bağlayıcı ve starter olarak kullanıldı.

Bulgular: Bu GAG seçici polimerler, çelik (4.6 × 1.6 mm) kolonlara basınçlı akışla dolduruldu, daha sonra baskılı GAG'lar çıkarıldı ve her bir miligram polimerin bağlanma kapasitesini hesaplamak için analiz edildi. GAG seçici sütunlarının kalibrasyon eğrileri 62.5 ng/mL ila 1000 ng/mL'den az% 5 katsayılı varyasyon ve daha düşük matriks etkisinden elde edildi.

Sonuç: Baskılı kolonlarımız, farklı GAG'ları idrardan seçici ve hassas bir şekilde ayırdı. Matriks etkisi, minimum düzeyde görülmüştür.

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Introduction

Glycosaminoglycans (GAGs) are the most abundant linear heterosaccharides in the body, which bind to proteoglycans [1]. According to their sugar residues, GAGs are called as hyaluronic acid, chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HSA) [2–4]. They are classified their isomeric structure of three monomeric repeating amino sugar units such as N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and uronic acid (UA). CS and DS are composed of GalNAc and uronic acid, which bind via β(1–4) or β(1–3) linkages. This results in CS and DS being stereoisomers with the same mass, only difference is a carboxyl group orientation. Moreover, there are also sulfate groups, which are negatively charged, and provides negative properties. These characteristic structures enable GAGs to form stable gel matrix structure by attracting water to resist pressure, which presses cells, tissues or organs. The importance of these polymers are proven by their accumulation or deficiencies to reveal different diseases. The disorders of the GAGs were classified as genetic deficiencies of lysosomal GAG hydrolase enzymes, which result in the accumulation of GAG molecules in lysosomes, a group of diseases called mucopolysaccharidosis [5]. Mucopolysaccharidosis shows a variety of symptoms (skeletal distortions, macrocephaly, speaking and learning failures, abnormal hair development, corneal blurring, hepatosplenomegaly, pneumonia, and mental retardation) that effect multiple organ involvement [6–10]. GAGs are important for the detection of different mucopolysaccharidoses, and also for diagnosis and follow-up of osteoarthritis [11], rheumatoid arthritis [12] and some cancers [8]. Thus, their determination is very important for diseases and the metabolism. In chromatographic techniques, same mass ratio and chemical structure is a challenge to overcome separation problems or further extraction techniques must be used. Currently, there are chromatographic [13, 14], spectrophotometric [15–17] and electrophoretic [18] techniques for urine GAG detection. Sometimes, it is hard to detect these sugars selectively, some of them superimposed or hard to separate, they are highly polar and micro heterogeneous; negatively charged sulfate, hydroxyl, carboxylic acid and acetyl groups cause the polarity, and the presence of different groups such as acetyl groups causes the microheterogeneity [19].

In this study, we developed CD and DS molecularly imprinted copolymers for GAG selective separation from urine. The selectivity of the molecular imprinting technology provided affinity column manufacturing and sensitivity of the mass spectroscopy was useful to characterize the system.

Materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich (USA) and all the solutions were chosen as MS grade. Steel chromatography columns were obtained from Waters and emptied their filling materials to fill MIPs (4.6×1.6 mm). Mass spectrometry measurements were carried out in Tandem Quadrupole Xevo TQD Tandem MS systems. Urine samples were collected with the approval of Ege University Clinical Researches Ethical Committee (permit 14/05/2015 15-4.1/17) from children of 0–3 year-old healthies to obtain high GAG excretion [20]. Parents of the children were informed about the purpose of the study and permission was obtained by signed forms. Urine samples (24 h urine-all the urine excreted over a 24-h period) were collected and pooled to obtain more natural GAG in urine from children who did not have any kidney diseases, idiopathic hypertension, urinary system infection or type-1 diabetes.

Methods

Optimization of standard glycosaminoglycans in tandem mass spectrometry

Each GAG fragment was degraded in a 3 M methanolic HCl solution (GAG concentration 1 mg/mL) at 65°C for 75 min, then dried with nitrogen gas for mass spectrometry detection. The degraded GAG units were dissolved in 10 mM dehydrated ammonium acetate methanol solution [7] for MS/MS analysis to find m/z ratios of GAG fragments. For mass spectrometry measurements, different fragments were directly infused into the mass detector without any chromatography column and characterized in positive ion mode (ESI+) in Multi Reaction Monitoring (MRM) mode.

Chromatographic column material preparation, filling process into steel column and testing

Column material was prepared as follows; 1 mg GAG (CS and DS separately) was dissolved in asetonitrile,

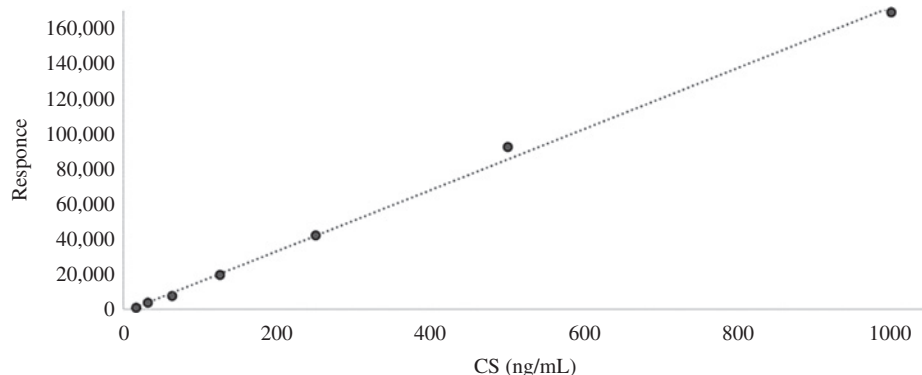**Table 1:** Characterization of the fragmented GAGs by mass spectrometry.

Glycosaminoglycan	Parent ion	Daughter ion
Chondroitin sulfate	394.2 (Cone: 40)	138.1 (Coll: 31)
Dermatan sulfate	448.2 (Cone: 53)	258.2 (Coll: 30)
Heparan sulfate	663.5 (Cone: 60)	551.3 (Coll: 27)

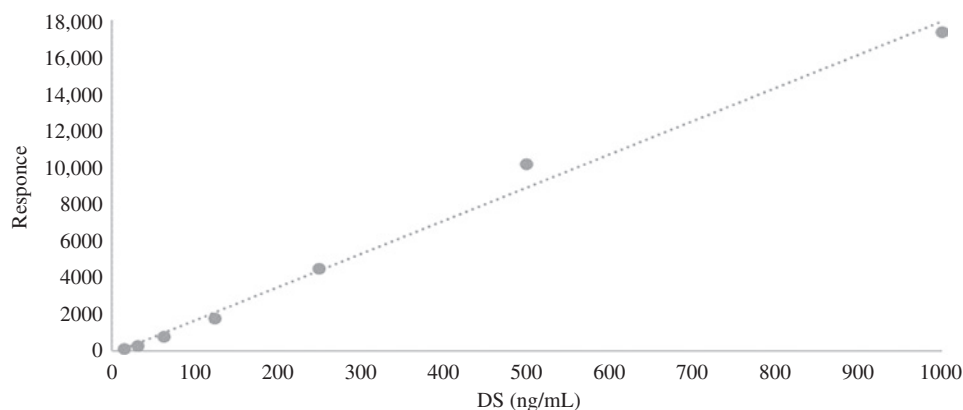The m/z ratio and cone voltages of parent ions and collision energy voltages of daughter ions are listed.

respectively. For each GAG, 4 mg acrylamide, 1 mg AAPBA, 900 µL trimethylolpropane trimethacrylate as a cross-linker, and 10 mg azobisisobutyronitrile as an

initiator were mixed in flask to synthesize molecularly imprinted polymers by using precipitation polymerization in 40 mL acetonitrile. The polymerizations were carried out at 65°C degrees for 18 h as a thermo-radical polymerization [21]. One polymer was prepared without GAG to serve as a control polymer, non-imprinted polymer (NIP). Polymers were dispersed in acetonitrile and dried. Then the polymers were filled into the steel column from outlet of the column towards inlet by using cylindrical metal probe to press polymers. After filling process, column was employed to a HPLC; filling process was carried out until the backpressure was not observed. The extraction of GAGs from the imprinted solid phase

A
Compound: Chondroitin sulphate derivative
Compound m/z: 394.2>138.1
Coefficient of Determination: 0.9972
Calibration Curve: y = 173.19x − 1143.1
Response Type: External standart area

B
Compound: Dermatan sulphate derivative
Compound m/z: 448.2>258.2
Coefficient of Determination: 0.9919
Calibration Curve: y = 18.068x − 147.17
Response Type: External standart area

Figure 1: (A, B) Calibration curves.
These calibration curves were prepared with GAG concentrations between 62.5 ng/mL and 1000 ng/mL. Response areas were proportional to concentrations. As a standard equation of the calibration curve ($y = mx + n$), y represents response, x represents the concentration of GAGs ([DS], [CS]). (A) MRM response areas of CS's 138.1 daughter ion of the 394.2 parent ion. (B) MRM response areas of DS's 258.2 daughter ion of the 448.2 parent ion.

polymers was achieved by methanol:acetic acid (4:1) solution at a flow rate of 300 μL/min.

Results and discussion

MS optimization and production of GAG fragments

All fragments were ionized in ESI +. The degraded GAG fragments which were dissolved in 10 mM anhydrous ammonium acetate were infused directly into the mass spectrometer to monitor m/z values of the fragments. Ionization parameters were set by considering the maximum peak energy of the fragments in the mass detector, as follows: 2.7 eV capillary voltage, 350°C desolvation temperature, and 650 L/h desolvation gas flow rate. Individual parameters of the fragments in MRM and their collision energy levels are given in Table 1. MS/MS optimization for the concentration of fragments of each GAG was carried out by using the retention time in the mass detector and measuring the response area of mass chromatograms.

From the MS/MS results, three calibration curves were prepared for each GAG concentrations <1000 ng/mL (Figure 1A and B) and GAG concentrations in samples were later calculated using these calibration curves.

SPE column filling and GAG elution

In the polymer synthesis process, we primarily considered the sulfate, carboxyl and hydroxyl groups of GAGs when choosing acrylamide, a basic-neutral monomer to attract negatively charged groups and AAPBA, which has boronic acid residue to attract sugars via diol bond formations [22]. Because of the high molecular mass of GAGs, a surface imprinted polymerization technique was chosen to prevent in case of GAGs would be embedded inside the polymers. Therefore, precipitation polymerization was chosen to form uniform spheres as the GAG imprinted polymer shapes [23]. The elasticity and uniform structure of the spheres make them suitable for high pressure chromatographic separations. It was also easy to perform the column filling process because of this elasticity and uniformity of the material. Polymers were investigated by FTIR to characterize the polymer characteristics (Supplementary Figure 3). The green curve is the GAG imprinted polymer, ~3500 represents the OH stretching, ~2900 represents aliphatic C-H stretching. As

it can be seen in supplementary data, OH bond signals were decreased because the imprinting process has occurred via hydrogen bonds. The pale blue represents, GAG-removed polymer, 3500 OH stretching signal was increased because of the free OH groups. ~1720 cm^{-1} for sugars bound to the phenylboronic acid residues and C=O ester peaks. Pale blue represents CS and DS, purple curve represents GAG removed polymer, where it can be seen OH stretching, the red is only acrylamide polymer and dark purple is control polymer. After polymerization process the polymer-filled steel columns were tested under pressurized liquid chromatography system until the flow rate was steady. Before applying the extraction solvent, first acetonitrile and then absolute methanol were flowed through the column. Methanol that was collected from the outlet side of the column was examined for GAG content, and MS/MS results showed that GAGs were not removed from the polymer by using methanol. We then infused methanol:acetic acid (4:1) through the MISPE column to remove the individual GAG. GAGs (CS, DS and HS) were eluted and chemically degraded for MS/MS analysis.
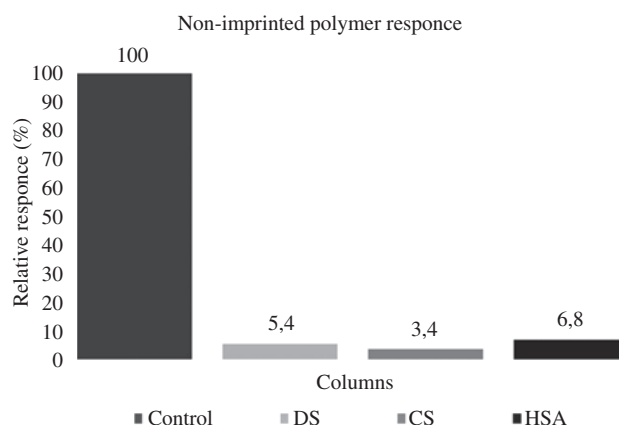

Figure 2: Comparison of relative responses from non-imprinted and molecularly imprinted polymers.
Non-imprinted polymer is used as a control polymer to measure the separation specificity of the imprinted polymers. In the eluent solution of methanol DS, CS and HSA were recovered less than 7%, which indicated that imprinted polymers had specific affinity to GAGs.

Table 2: Comparison of coefficient variations (CV) of lowest concentrations to assess interdays and intradays.

Glycosaminoglycan (62.5 ng/mL)	Interday (n = 7) CV%	Intraday (n = 3) CV%
Chondroitin sulfate	3.4 ± 0.84%	3.89 ± 0.89%
Dermatan sulfate	1.4 ± 0.71	2.3 ± 0.77
Heparan sulfate	5.06 ± 1.14	5.86 ± 1.94

MISPE characterization and urine sample applications

Column selectivity, repeatability and limit of detection

Urine samples were collected between 0 and 3 years of age (n = 10) for higher level of glycosaminoglycans in urine and concentrated [6]. The samples were pooled to perform same concentration of samples to analyze the methods optimization parameters. These urine samples were applied to the non-imprinted columns to examine whether they contained any fragments with the same m/z ratio(s) as GAG standards, as it is shown in Figure 2, after MeOH:AcCOOH application, the chemically degraded extractions were investigated by MS/MS analysis and no response was obtained for column affinity (Figure 2). Two different imprinted polymers of CS and DS were compared to NIP polymer and retention times were given in Supplementary file (Suppl. 1.). This comparison was carried out by analyzing outlet samples of the prepared column, before extraction process.

After examination of selectivity, 62.5 ng/mL standard samples (n = 10) were applied to the columns to examine column performance tests. In a 300 μL/min flow of MeOH:AcCOOH (4:1), GAG eluents were analyzed for MS/MS analysis (Table 2).

The reusability of the imprinted columns was examined and the results showed that reproducibility was good (Figure 3A and B); coefficient of variation (CV) results were 5.0% and 3.9%, respectively, for the CS and DS.

The amount of glycosaminoglycans that bound to the polymers (Q%) was defined according to the equation;
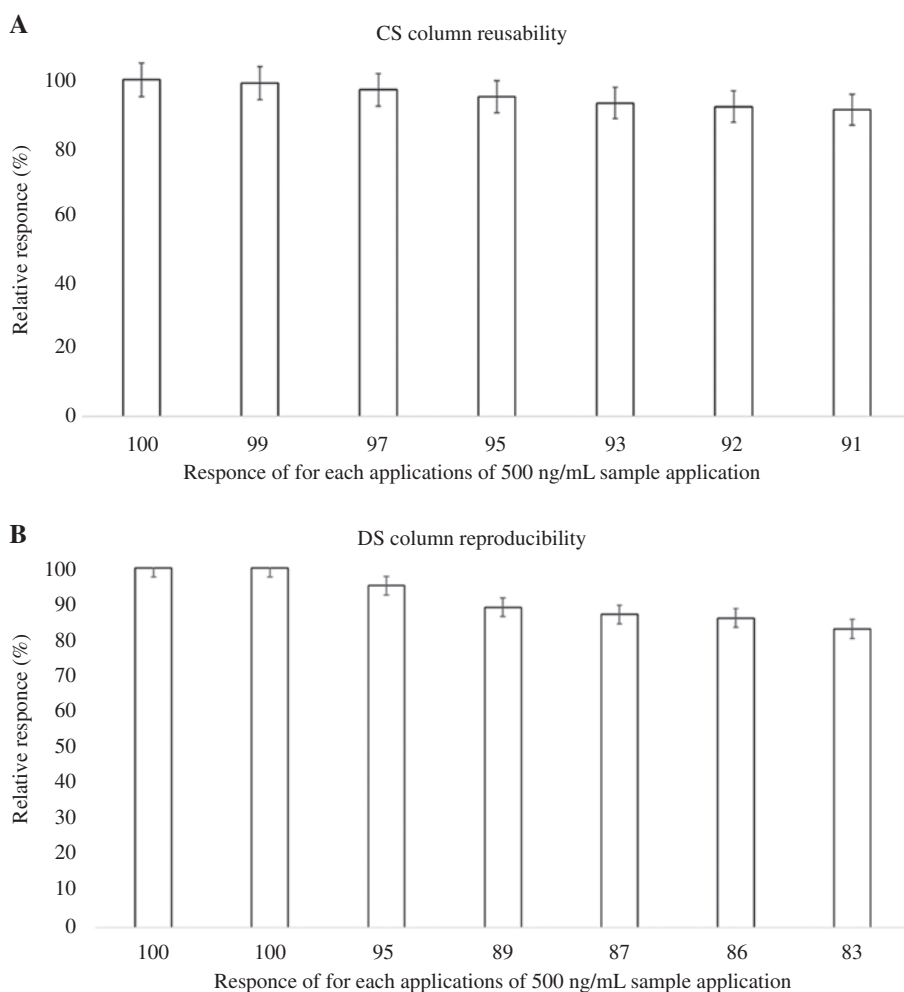
$$Q\% = \frac{C_0 - C_s}{C_s}$$

A

B

Figure 3: (A, B) The reusability of molecularly imprinted polymers for every 500 ng/mL GAG application consequently. (A) CS imprinted column material reusability capacity. (B) DS imprinted column reusability capacity.

where C_0 is the initial concentration of sugar and C_s is the concentration of glycosaminoglycan in the urine after rebinding. Q_{max} is and Q was calculated as 332 ng/mL for 500 ng/mL DS: 332 ng/mL, CS: 432 ng/mL equilibrium constant. 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, KD is the equilibrium dissociation constant and $[C]$ is the equilibrium constant of template [24]. K_d was calculated as for DS = 0.016 and CS = 0.0123. In most clinical mass spectrometry-chromatographic techniques, internal standards (IS) are used to correct for the loss of analyte during sample preparation or sample inlet. For this process, a known amount of IS was added to every sample, both calibrators and unknowns to base the calibration on the ratio of response between the analyte and the IS (by calculating response factor.) instead of basing it on the absolute response of the target analyte. However, the requirements of the IS (that it should be very similar to the analyte with a similar retention time and similar derivatization as well as it should be stable, not interfering with the sample components) to be selected is often a challenge. In this study, chemically degraded GAG fragments were added to see matrix effect in spiked urine. In spiked urine samples the matrix effect was found to be <5% and real sample GAG concentrations calculated by using this data (Table 3). This indicates that in our method, it is not necessary to use an IS (which means we can avoid the problem that chemically degraded fragments would have to be examined by NMR for IS synthesis).

Table 3: GAG standard adding in urine and column separation characteristics based on the column separation capacity.

GAG column	Added GAG	Detected GAG (matrix effect)	GAG in urine
Spiked urine	500	504.2	
Chondroitin sulfate	500	681.5	177.25
Dermatan sulfate	500	514.2	12
Heparan sulfate	500	547.21	43.01

Spiked urine was concentrated by adding 500 ng/mL standards and then directly applied to the columns to monitor matrix effect and real sample application for remove matrix effect by comparing spiked urine (all units in ng/mL).

Conclusion

In this study, we developed chromatographic affinity columns that can specifically separate CS and DS. Specific columns for each of these GAGs were designed using MIT. Easy and economic production and the uniform structure of the imprinted microspheres developed are advantages of this novel method of column production. The selection of acrylamide as monomer which can be made more selective of sulfated and negatively charged GAGs increased the selectivity of the column filling material. AAPBA was used to increase the specificity of the molecular imprinted columns because its functional groups can attract sugar groups of GAGs. To check the effectiveness of the columns, they were analyzed by an MS/MS system and our results show that they work with high selectivity. This study showed that columns produced by MIT enhance GAG selectivity in urine GAG determinations. Through this process, GAG separation from urine is simplified, accelerated and made more specific and economic.

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