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Note

High-performance liquid affinity chromatography of nucleosides, nucleotides and carbohydrates with boronic acid-substituted microparticulate silica

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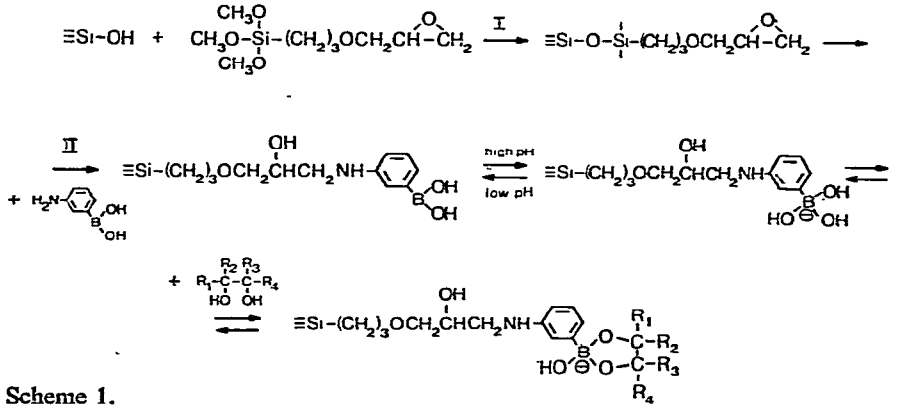
During the last decade the use of boronic acid-substituted polymers for the separation of diol-containing molecules such as nucleosides, nucleotides, catecholamines, carbohydrates and transfer RNA has been well documented[1–7]. The vicinal diols of these substances are able to form reversibly cyclic boronate esters with the boronate anion at high pH (see Scheme 1), and the formation of these complexes is dependent on pH, ionic strength, temperature and, in the case of nucleic acid components, on the structure of the base[1]. The polymers used for the preparation of these separation media, *e.g.*, cellulose and polymethacrylic acid, all possess the disadvantage of poor flow-rate and limited resolution because of their low rigidity and large bead size. They have hitherto been used in conventional (low pressure) liquid chromatography, for instance in the separation of catecholamines in urine[8], and as group-



Scheme 1.

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specific ligands for the isolation of nucleotides from urine and plasma, of interest in the diagnosis of certain diseases[9–11].

Recently a technique has been reported, termed high-performance liquid affiinity chromatography (HPLAC), which combines the speed of analysis inherent in HPLC with the specificity of affinity chromatography[12]. In the systems studied, "biomolecules" such as inhibitors or antibodies were bound to the supports. In the present paper we describe an extension of the HPLAC technique using the non-biological affinity ligand boronic acid. The latter was bound covalently as 3-aminobenzene boronic acid to small, 10 μm, porous silica particles (see Scheme) and used in the separation of mixtures of nucleosides, nucleotides or carbohydrates.

EXPERIMENTAL

Chemicals

Porous silica (LiChrosorb Si 100, 10 μm) was obtained from E. Merck (Darmstadt, G.F.R.), 3-aminobenzene boronic acid hemisulphate from Aldrich (Milwaukee, WI, U.S.A.) and γ-glycidoxypropyltrimethoxysilane (Silane Z-6040) from Dow Chem. (Midland, MI, U.S.A.). Nucleosides and nucleotides were from Sigma (St. Louis, MO, U.S.A.), with the exception of 3′,5′-cyclic adenosine monophosphate which was from Serva (Heidelberg, G.F.R.). All solvents and other chemicals, obtained from commercial sources, were of analytical grade and used without further purification.

Synthesis of epoxy-substituted silica (Scheme 1, reaction I)

The coupling procedure used was a modification of an earlier method[13]. All equipment was rigorously dried. To porous silica (3 g), dried at 200°C overnight and then slurried in 100 ml of sodium-dried toluene, were added 80 μl of triethylamine (potassium hydroxide-dried) and 4 ml of γ-glycidoxypropyltrimethoxysilane. The mixture was refluxed under nitrogen for 4 h in a flask provided with a PTFE rod stirrer. Then the substituted silica was filtered off and washed on a glass filter-funnel with toluene, acetone and diethyl ether (100 ml of each) and finally sucked dry. After hydrolysis of epoxy groups to diols with dilute sulphuric acid (pH 2, 90°C, 1 h), the concentration of free epoxy groups was determined by periodate oxidation[14] and was found to be about 320 μmol per g silica.

Synthesis of boronic acid silica (Scheme 1, reaction II)

3-Aminobenzene boronic acid (600 mg) was added to a suspension of epoxy-substituted silica (2 g) in 15 ml of water. The pH was then adjusted to 8.5 with 3 *M* sodium hydroxide and the mixture was placed in an ultrasonic bath for 10 min to facilitate dissolution of boronic acid. The pH was again adjusted to 8.5 and the coupling reaction was conducted at 21°C for 24 h with gentle shaking. The reaction product was filtered off and washed thoroughly with 0.5 *M* sodium chloride, 0.1 *M* sodium bicarbonate, 1 m*M* hydrochloric acid, water and acetone, and finally dried under vacuum. The boron content[15] of the boronic acid-substituted silica was 0.27% (w/w) corresponding to 250 μmol per g silica.

Chromatography

An Altex Model 110A pump (Altex, CA, U.S.A.) was used together with an

Spectro Monitor III UV-detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) or a Model Optilab Multiref refractive index detector (Optilab, Vällingby, Sweden).

The boronic acid silica (about 1.2 g) was packed in a polished 316 stainless-steel column (100 × 5 mm I.D.; total volume, 2.0 ml) with the upward slurry packing technique[16] in water–methanol (10:90, v/v) at 14 MPa (2000 p.s.i.). The performance of the column was estimated with a mixture of phenol, 2,6-dimethylphenol and 4-*tert.*-butylphenol in water–methanol (50:50, v/v) and a typical HETP value was 0.083 mm (4-*tert.*-butylphenol) corresponding to a theoretical plate number of about 1200.

All chromatographic procedures were performed at room temperature (20–22°C) and the pressure was about 2.1 MPa (300 p.s.i.) at a flow-rate of 1 ml/min. An injection volume of 10 μl containing 10–50 μg of each component in the mixtures was used throughout the experiments. The interstitial volume (void volume) of the column was 1.4 ml as indicated by the peak obtained from ^2H_2O.

RESULTS AND DISCUSSION

Preparation of epoxy-substituted silica

Chemical coupling of epoxysilane to the surface of porous silica can result in cross-linking and polymerization, if traces of water are present during the reaction. The epoxysilylation was performed under anhydrous conditions in order to minimize the extent of these undesirable side reactions. It must be emphasized that it is of considerable importance to establish a thin molecular coating on the silica particles. If the surface layers are too thick, caused by extensive polymeric networks, low chromatographic efficiency may result, due to slow solute diffusion, especially for macromolecules such as proteins and polynucleotides.

A reference column of epoxy-substituted silica not reacted with 3-aminobenzene boronic acid did not show any retardation or adsorption of the substances studied. Furthermore, the recovery of many proteins was nearly quantitative on the reference column, *e.g.*, with human serum albumin about 98% of a 10-μg sample was unretarded.

Separation of nucleosides

The use of boronic acid-substituted cellulose in conventional (low pressure) chromatography, allowing the separation of complex mixtures of nucleosides, is a well-known technique[1]. The chromatogram in Fig. 1 demonstrates a separation with HPLAC of a synthetic mixture of eight nucleosides applied to a column with boronic acid silica. Isocratic elution was performed with 0.1 *M* sodium phosphate, pH 7.5, and most of the components were well separated. As expected, the nucleosides having only one free hydroxyl group in position 3 of the ribose moiety, *i.e.*, the deoxynucleosides and thymidine, are less retarded than the others. Although these compounds cannot form cyclic boronate complexes with the solid support ,retardation and separation are observed. This phenomenon is probably caused by hydrophobic interactions between the benzene rings of the substituted silica and the bases of the nucleosides, possibly together with some contribution from hydrogen bonding. In addition, some complex formation between single hydroxyl groups and the boronic

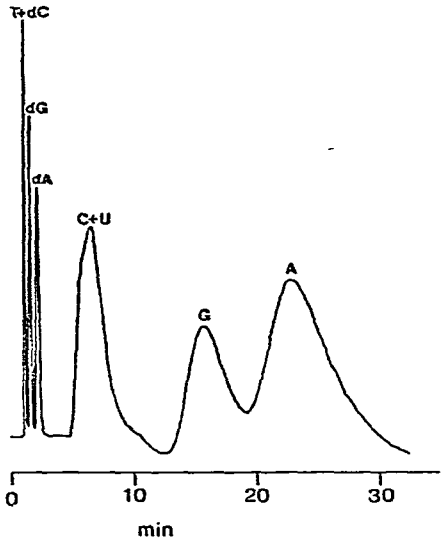

Fig. 1. Separation of nucleosides with boronic acid silica. Sample: a mixture of adenosine (A), deoxyadenosine (dA), cytidine (C), deoxycytidine (dC), guanosine (G), deoxyguanosine (dG), thymidine (T) and uridine (U). Eluent: 0.1 *M* sodium phosphate, pH 7.5; flow-rate, 2 ml/min. Detection: UV at 260 nm.

acid on the support is possible. At the selected pH, separation was complete within 35 min, compared with more than 30 h for a comparable mixture applied to boronic acid cellulose[1].

Separation of nucleotides

Separation of nucleotides with anion exchangers in HPLC systems has been reported[17,18]. Boronic acid silica can be used to separate nucleotides by employing another mechanism. Fig. 2 shows the complete separation of the mono-, di- and

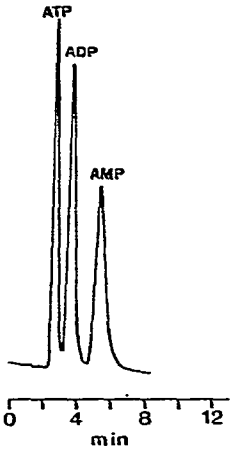

Fig. 2. Separation of nucleotides with boronic acid silica. Sample: a mixture of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Eluent: 0.1 *M* sodium phosphate, pH 7.5; flow-rate, 1 ml/min. Detection: UV at 260 nm.

triphosphates of adenosine. The structures of these compounds differ only in the number of phosphate groups attached to the carbon at position 5 of the ribose moiety. It is seen that the number of negatively charged phosphate substituents markedly influences the ability to form boronate complexes at a fixed pH. Adenosine monophosphate and 3',5'-cyclic adenosine monophosphate can be completely separated in the same system.

Separation of carbohydrates

Carbohydrates can be separated chromatographically in a number of ways, including gas, paper, thin-layer and ion-exchange chromatography. More recently HPLC has become an established technique for the separation of carbohydrates[19–21]. The use of a poly(4-vinylbenzene boronic acid) resin to separate fructose and glucose in an "open column" system has been reported[22]. Unfortunately this method suffers from long separation time and poor resolution.

A system is described here for rapid separation of carbohydrates using the same affinity support as for nucleic acid components. As shown in Fig. 3, fructose and glucose are completely separated on boronic acid silica.

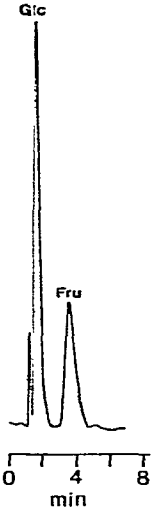

Fig. 3. Separation of carbohydrates with boronic acid silica. Sample: a mixture of glucose (Glc) and fructose (Fru). Eluent: 10 m*M* sodium pyrophosphate, pH 8.25; flow-rate, 1 ml/min. Detection: refractive index.

In Table I the capacity factors for some carbohydrates and related compounds, as determined on boronic acid silica by separate injections, are presented. It is seen that compounds having the most favourably oriented hydroxyl groups, in terms of ability to form a boronate complex with the solid support, have the largest elution volumes. Thus, glucose and sucrose are eluted early, whereas sorbitol and mannitol possessing several vicinal *cis*-diols are retarded. In the elution of some of the carbohydrates (fructose, mannitol and sorbitol) the refractive index detector gave negative

TABLE I

CAPACITY FACTORS FOR CARBOHYDRATES AND RELATED COMPOUNDS ON BORONIC ACID SILICA

Eluent: 10 m*M* sodium pyrophosphate, pH 8.25; flow-rate, 1 ml/min. Detection: refractive index.

Carbohydrate	*Capacity factor (k')*
Glucose	0.14
Sucrose	0.21
myo-Inositol	0.29
Galactose	0.79
Fructose	2.86
Mannitol	3.79
Sorbitol	4.14

responses, and in the experiment shown in Fig. 3 the polarity of the observed signal was reversed after the appearance of glucose.

Other separations

The chromatography of synthetic mixtures of compounds able to bind to boronic acid silica, as presented in this paper, indicates the versatility of this chromatographic medium. This is further illustrated by our finding that transfer RNA can bind at high pH and then be eluted at low pH.

If a substance has a tendency to bind very strongly to boronic acid silica it is advantageous to add to the eluent a small amount of a "counter ligand" that also can bind reversibly to the solid support, thus making the substance of interest elute earlier, resulting in shorter separation times and decreased broadening of the peaks. This technique has been used in some preliminary experiments to separate crude mixtures of nicotinamide–adenine dinucleotide (NAD) and synthetic bi- and trifunctional derivatives made up from NAD molecules connected with a "spacer"[23], thus suggesting the use of boronic acid silica in synthetic chemistry.

Preliminary results have also shown that boronic acid silica can be used to separate nucleosides present in human urine. A crude sample (2 ml) was applied directly to the column without pre-treatment other than pH adjustment. After removal of non-interacting material, the retarded substances were separated with a decreasing pH gradient. The sensitivity was considerably improved by the use of the large sample volume.

CONCLUSIONS

Chromatography on boronic acid silica drastically reduces the separation time of diol-containing substances without loss of resolution when compared to less rigid polymers such as cellulose and polymethacrylic acid. This is important, especially in routine analytical procedures. However, compared to other HPLC materials, the resolution of boronic acid silica seems to be limited by the slow attainment of equilibrium between ligand and solute. On the other hand, it is far more selective than some HPLC materials. We feel that this new stationary phase may find applications in a number of areas for the separation of substances of biological importance such as catecholamines and glycoproteins.

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