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BORONIC ACID–SILICA: A NEW TOOL FOR THE PURIFICATION OF CATECHOLIC COMPOUNDS ON-LINE WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The properties of silica-immobilized benzeneboronic acids are discussed both in terms of coating of the silica and efficiency of these particles for use in a high-performance liquid chromatography column. The silica derivative displays an affinity for catecholic compounds, whose content in biological samples may be estimated with a column-switching system using boronic acid silica on-line followed by a reversed-phase chromatographic system. The dual system is suitable for direct injection of crude biological samples, and at least 1 ml of sample can be injected without additional band broadening.

INTRODUCTION

The analysis of catecholic compounds in biological material has been the subject of several studies in recent years. The development of the electrochemical detector allowed for analysis of normal tissue extracts with high-performance liquid chromatography (HPLC)[1]. Today several catecholic compounds are routinely determined analytically and in large series[2]. The chromatographic separations are fast and the methods are well established. However, for most biological samples such as urine, serum and tissues, a purification step is necessary before introducing the sample into the HPLC system. This purification step has, for the past twenty years, been performed mainly on activated alumina[3]. The selective adsorption of *cis*-diols on alumina, performed column- or batch-wise, provides concentrated and purified samples suitable for subsequent analysis on the analytical column. However, this treatment introduces uncertainty and furthermore is time consuming.

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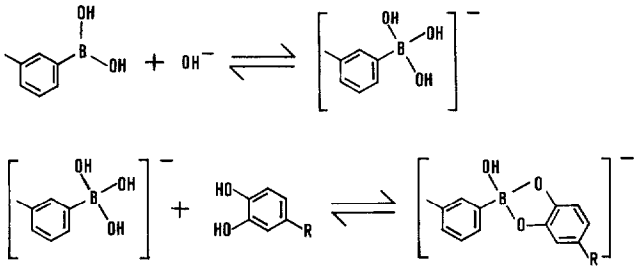
Derivatives of boronic acid also form complexes with *cis*-diols (Scheme 1). The usefulness of this property with respect to catecholic compounds has been previously shown[4,5]. However, the boronic acid gel used in these reports was not suitable for high-pressure techniques. This problem was overcome by immobilizing boronic acid derivatives on microparticulate silica, providing a packing material useful for high-performance liquid affinity chromatography (HPLAC)[6,7].



In this study we introduce boronic acid–silica into the field of catecholic compounds. The properties of the gel are studied and its usefulness shown in some applications.

EXPERIMENTAL

Chemicals

Porous silica (LiChrosorb Si 100, 5 μm) was obtained from E. Merck (Darmstadt, G.F.R.), 3-aminobenzeneboronic acid hemisulphate from EGA-Chemi (Albuch, G.F.R.), γ-glycidoxypropyltrimethoxysilane (silane Z-6040) from Dow Chemicals (Midland, MI, U.S.A.) and 3,4-dihydroxyphenylacetic acid (DOPAC) from Fluka (Buchs, Switzerland). Two buffer–methanol systems were used. System A consisted of 0.1 *M* sodium phosphate (pH 7.5)–methanol (90:10) and system B consisted of 0.1 *M* sodium formate (pH 3)–methanol (90:10).

All other solvents and chemicals, obtained from commercial sources, were of analytical grade or HPLC grade and were used without further purification. All aqueous solutions were prepared from double-glass-distilled water.

Synthesis of epoxy-substituted silica

The coupling procedure used was a modification of a method previously described[6]. Porous silica (15 g) and all experimental equipment were dried at 220°C overnight. Sodium-dried toluene (300 ml), potassium hydroxide-dried triethylamine (450 ml) and γ-glycidoxypropyltrimethoxysilane (12 ml) were slurried together with the silica under nitrogen in a flask provided with a glass rod stirrer. When the mixture had been refluxed for 5 h an additional 10 ml (and after a further 15 h another 5 ml) of γ-glycidoxypropyltrimethoxysilane were added to the reaction mixture. The total reaction time was 24 h. The epoxy-substituted silica obtained was filtered, and washed in succession with toluene, acetone and diethyl ether and sucked dry under vacuum.

A small amount (1 g) of the epoxy-substituted silica was hydrolyzed in 20 ml of 0.1 *M* sulphuric acid–sodium hydroxide buffer (pH 2, 50°C, 5 h). The concentration of diol groups was determined by periodate oxidation[8] and was found to be 510 μmol

per gram of substituted gel. The elemental analysis of the hydrolyzed gel dried in vacuum gave C = 6.43% and N = 0.07% (mean of two determinations).

Synthesis of acetylated boronic acid–silica

Epoxy-substituted silica (7.3 g) was added to 1.8 g of 3-aminobenzeneboronic acid hemisulphate dissolved in a minimum volume of water (an ultrasonic bath was used to facilitate the dissolution). The total volume of the mixture was 32 ml. The pH was adjusted to 7.0 with 5 *M* sodium hydroxide. The coupling reaction was conducted at 21°C for 20 h. The boronic acid–silica was filtered and washed thoroughly with water, methanol, water, 2 *M* sodium chloride in water, methanol and diethyl ether, and finally sucked dry under vacuum.

In order to hydrolyze residual epoxy groups, 7.3 g of boronic acid–silica were slurried in 60 ml of 0.1 *M* sulphuric acid–sodium hydroxide buffer (pH 2, 21°C, 15 h). The boronic acid–silica was then filtered and washed with 0.1 *M* sodium phosphate (pH 7.0), water, methanol and diethyl ether, and dried under vacuum. Finally the boronic acid–silica (7.0 g) was acetylated in 25 ml of potassium hydroxide–dried pyridine containing 4 ml of acetic anhydride. The mixture was gently shaken for 6 h at 21°C. The acetylated gel was filtered and washed thoroughly with pyridine, methanol, 0.1 *M* sulphuric acid–sodium hydroxide buffer (pH 2), water, methanol and diethyl ether. The elemental analysis of the acetylated boronic acid–silica (ABA-silica) dried in vacuum gave C = 9.77% and N = 0.57% (mean of two determinations).

Chromatography

Two Altex Model 110A pumps (Altex, Berkeley, CA, U.S.A.) were used together with a Rheodyne sample injector Model 7120(A) and a Rheodyne switching valve Model 7010(B) (Rheodyne, Cotati, CA, U.S.A.). A thin-layer amperometric detector, Model LC-2A, was used (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The glassy carbon electrode was operated at +0.75 V *versus* an Ag/AgCl reference electrode. A SpectroMonitor III UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was also used.

The ABA-silica was packed in a polished 316 stainless-steel column (100 × 5 mm I.D.) with the upward slurry packing technique[9] in chloroform–methanol (2:1) at 2100 p.s.i. The packing of the column was checked in methanol–water (1:3) by the injection of 2,6-dimethylphenol, which gave at theoretical plate number of *ca.* 3000. The reversed-phase column was packed with Nucleosil C_{18} (5 μm, Macherey, Nagel & Co., Düren, G.F.R.) in a polished 316 stainless-steel column (200 × 5 mm I.D.) according to a technique described elsewhere[10]. The performance of the reversed-phase column was estimated in methanol–water (7:3) by the injection of 4-*tert.*-butylphenol, which gave a theoretical plate number of *ca.* 8000.

RESULTS AND DISCUSSION

Characteristics of the ABA-silica

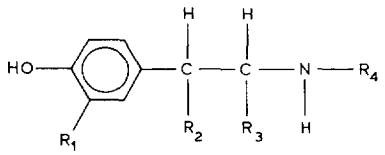
When preparing epoxy-substituted silica it is desirable to get a thin coating that covers the surface. If water is present during the silanization process, a thick polymer layer may be the result. This can cause slow diffusion of the solute, which diminishes the efficiency of the column. For this reason the silanization was performed under

anhydrous conditions. It has been shown that amines catalyze the reaction between alkoxysilanes and silanol groups[11], and di-*n*-butylamine has been used in the preparation of chemically bonded stationary phases from trimethoxysilanes[12]. The elemental analysis of our epoxy-substituted silica showed traces of nitrogen. This might imply that triethylamine also reacts with the epoxy groups. A calculation based on the carbon elemental analysis of the epoxy-substituted silica estimates the concentration of γ-glycidoxypropylsilane groups to be *ca.* 840 μmol per gram of epoxy-substituted gel or 2.8 μmol/m^2 (specific surface area 300 m^2/g, according to the manufacturer), which is in agreement with values obtained from other methoxysilanes of approximately the same size[12]. However, the value obtained from periodate oxidation is only 510 μmol per gram of epoxy-substituted gel, which suggests that only *ca.* 60% of the epoxy groups are accessible for reaction. Calculations from the nitrogen elemental analysis of the ABA-silica estimate the concentration of immobilized boronic acid to be 357 μmol per gram of gel.

The ABA-silica shows great affinity for catechols. This can be seen in Table I, which lists capacity factors (k') for some compounds related to catecholamine metabolism. As expected, only compounds with free *cis*-diols, *i.e.* catechols, are capable of forming complexis with boronic acid and are thus retained to a greater extent on the ABA-silica. The influence of pH on the retention volume was also studied. The increased retention at higher pH is shown in Fig. 1 for DOPAC. The addition of 50% methanol to the mobile phase gave no major divergency. The upper pH limit at which the column can be used for a prolonged time is determined by the rate of dissolution of the silica particles. The manufacturer recommends avoiding pH values higher than 8.

TABLE I

CAPACITY FACTORS ON ABA-SILICA FOR CATECHOLS AND RELATED COMPOUNDS WITH THE GENERAL FORMULA:



Eluent, 0.05 *M* sodium acetate–phosphoric acid (pH 5.0); flow-rate, 1 ml/min; detection, UV at 254 nm.

Compound	R_1	R_2	R_3	R_4	*Capacity factor (k')*
Tyrosine	H	H	CO_2H	H	0.20
Tyramine	H	H	H	H	0.26
Octopamine	H	OH	H	H	0.17
Dopa	OH	H	CO_2H	H	1.90
3-O-Methyldopa	OCH_3	H	CO_2H	H	0.24
Dopamine	OH	H	H	H	3.31
3-O-Methyldopamine	OCH_3	H	H	H	0.31
Noradrenaline	OH	OH	H	H	2.79
3-O-Methylnoradrenaline	OCH_3	OH	H	H	0.17
Adrenaline	OH	OH	H	CH_3	3.62
3-O-Methyladrenaline	OCH_3	OH	H	CH_3	0.22

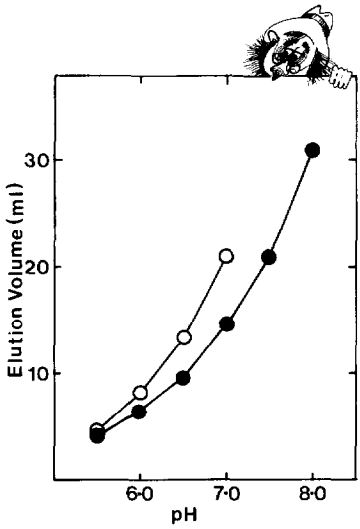


Fig. 1. Relationship between pH in the mobile phase and retention volume of DOPAC as measured from the injection point to the first noticed deflection from the baseline. Eluent: 0.1 *M* sodium acetate–phosphoric acid (pH 5.5) or 0.1 *M* sodium phosphate adjusted to the appropriate pH with 5 *M* sodium hydroxide, (●) with methanol (1:1) or (○) without methanol added to the mobile phase. Flow-rate, 1 ml/min; detection, UV at 254 nm.

Although the theoretical plate number for the ABA-silica column tested with 2,6-dimethylphenol in methanol–water (reversed-phase mode) is *ca.* 3000, the theoretical plate number is significantly lower (*ca.* 100) for the catechols in aqueous buffer systems (affinity mode), probably owing to a slow equilibrium between the boronic acid and the *cis*-diols of the catecholic compounds.

Recovery of compounds from the ABA-silica column was measured by injecting 100 μl of a solution of DOPAC (0.1 mg/ml). The column was washed with 10 ml (1 ml/min) of buffer A and then DOPAC was eluted by switching to buffer B. The eluate was collected and the amount of DOPAC eluted from the column was compared with the amount injected (diluted to the same volume). The determinations of the concentrations were made by analysis with the reversed-phase column, which estimated the recovery to be 99.1 % and 102.1 % for two separate runs.

About 150 injections, among them crude urine samples of 1400 μl, were made on the same ABA-silica column over several months without any noticeable deterioration of the column.

The column-switching system

The system used for direct injections is shown in Fig. 2. Samples (10–1400 μl) are injected with the sample injector (A) into buffer A and pumped (1 ml/min) via the switching valve (B) to the ABA-silica column. Compounds capable of forming complexes with boronic acid are enriched on the column while other compounds are eluted in the waste. The ABA-silica column is then washed with 10 ml of buffer A. The maximum volume of washing is dependent on the migration rate of the substance of interest. From Fig. 1 it can be seen that at pH 7.5 the maximum washing volume for DOPAC is *ca.* 20 ml, but in our experience 10 ml is enough to remove most

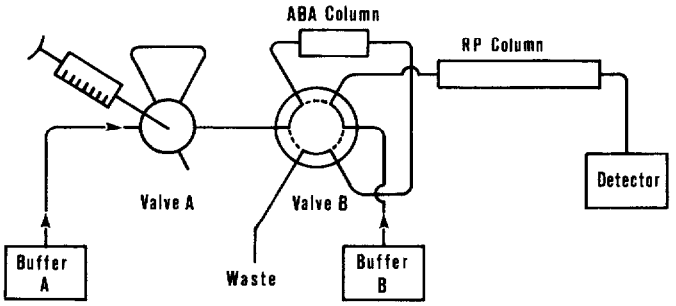


Fig. 2. Schematic arrangement of the column switching system. RP = Reversed-phase.

interfering substances. After the washing step the eluent is changed from buffer A to buffer B (pH 3.0) by the switching valve (B). The complexes between *cis*-diols and immobilized boronic acid are dissociated at once, and the eluted compounds are carried in a small volume to the reversed-phase column for further separation.

The additional band broadening of a 10 μl sample of DOPAC (0.1 μg/μl) caused by the ABA-silica column was 73%, compared with a direct injection of DOPAC on the reversed-phase column (k' = 4.6). This calculation was based on peak volumes of 0.90 ml and 0.52 ml, respectively. Even though the ABA-silica column causes some additional band broadening, no further broadening was noticed when the injection volume was increased from 10 μl to 1400 μl.

The reproducibility of the analytical system for three different samples, expressed as relative standard deviation, is shown in Table II.

Biological applications of the ABA–reversed-phase system

The versatility of the combined ABA–reversed-phase system was demonstrated by the analysis of DOPAC in human urine and in rat brain tissue. Urine was obtained from apparently healthy subjects. The samples were collected over sodium bisulphite, 10 mg/l (to prevent DOPAC from oxidation), and the only pretreatment performed was filtration through a Millipore filter (0.47 μm). Because the pH of the urine samples was between 6 and 7, no further adjustment was necessary. The determination of DOPAC content in one sample was made from a calibration curve as well as by the standard addition method. The results of the two methods are in accordance with each other, giving values of 1.16 μg/ml and 1.19 μg/ml, respectively. DOPAC levels

TABLE II

RELATIVE STANDARD DEVIATION OF THE ANALYTICAL SYSTEM FOR SAMPLES WITH DIFFERENT CONCENTRATIONS OF DOPAC

Sample	*Number of injections (n)*	*Relative standard deviation (%)*
DOPAC (1 μg/ml) in buffer B	10	3.5
Human urine (concentration of DOPAC *ca.* 1 μg/ml)	10	3.9
Human urine spiked to *ca.* 26 μg DOPAC/ml	8	1.6

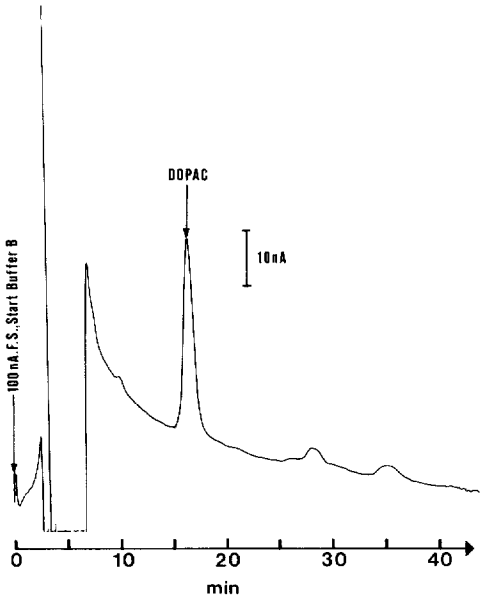


Fig. 3. Chromatogram of a urine sample (100 μl) from a healthy subject using the column switching system. Flow-rate, 1 ml/min; detection, amperometric operated at +0.75 V.

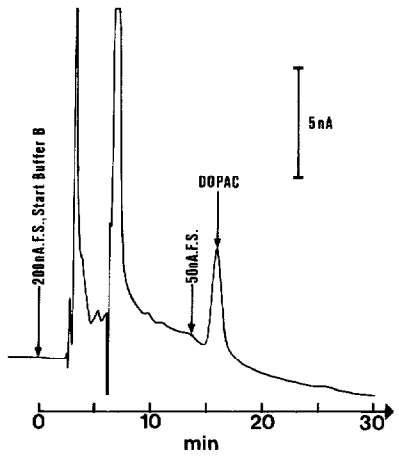


Fig. 4. Chromatogram of a homogenate (1400 μl) from a rat brain using the column switching system. Flow-rate, 1 ml/min; detection, amperometric operated at +0.75 V.

were also determined in urine collected over a 24-h period from two healthy subjects. The total amounts were found to be 0.64 mg and 1.08 mg, in agreement with estimates from other investigations[13,14]. Fig. 3 shows a chromatogram of a normal urine sample.

Analysis of DOPAC was also performed on rat brain tissue. Two whole brains from male albino rats (Sprague-Dawley) were weighed and homogenized, each in 3 ml of 0.4 *M* perchloric acid at 0°C. The homogenates were centrifuged at 30,000 *g* for 10 min and then filtered. Filtrates (3-ml aliquots) were adjusted to pH 6.5 by the addition of 3 ml of sodium phosphate buffer (pH 7.5) before 1400-μl volumes were injected. Fig. 4 shows one resulting chromatogram. The amount of DOPAC found in the two brains was 40.5 ng per gram of tissue and 56.8 ng per gram of tissue, respectively.

CONCLUSIONS

The boronic acid-silica prepared and used in this study has been shown to be an efficient tool for the isolation and enrichment of catecholic compounds in biological samples. It is possible to perform all steps at favourable pH values, contrary to earlier purification procedures. This property can be of great importance since some catecholic compounds (*e.g.* indolic catechols) are sensitive to both acidic and alkaline conditions.

The on-line technique described is suitable for automation and avoids the time-consuming alumina adsorption step. Furthermore, the precision of the analysis ought

to be improved when all steps are done on-line. The capacity of the ABA-silica is high enough to allow large amounts of sample to be injected. Because of immediate dissociation of catecholic compounds from the ABA-silica column at pH 2–3, it is possible to concentrate them and thus release a small injection volume to the analytical reversed-phase column. This enrichment of the sample makes the method highly sensitive. Obviously, the ABA column could also be combined with other HPLC columns, *e.g.* ion-exchange and size-exclusion columns, as useful alternatives in related applications.

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