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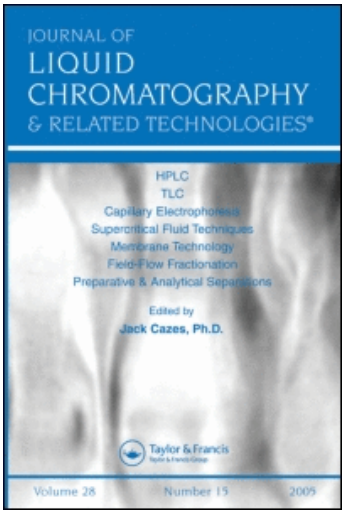

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Lee, H. G. and Jarrett, H. W.(1995) 'Glucuronic Acid-Silica, a Novel Support for High-Performance Cation-Exchange Chromatography', Journal of Liquid Chromatography & Related Technologies, 18: 6, 1113 — 1122
To link to this Article: DOI: 10.1080/10826079508009279
URL: http://dx.doi.org/10.1080/10826079508009279

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(6), 1113-1122 (1995)

GLUCURONIC ACID-SILICA, A NOVEL SUPPORT FOR HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Glucuronic acid was covalently coupled to aminopropyl derivatized silica by reducing the Schiff base with $NaCNBH_3$. This glucuronic acid-silica has a functional pKa in the pH 3-4 range and binds 202 mg hemoglobin per gram material at pH 5. The support is a high-performance cation-exchanger and exhibited only minor hydrophobic interaction with applied samples. It also gave reasonably constant retention of the proteins tested.

INTRODUCTION

Ion-exchange chromatography has been a powerful technique in purifying proteins based on their charge characteristics. Low-pressure ion-exchangers such as carboxymethyl-Sephadex have been widely used. Low-pressure ion-exchange chromatography, however, has limited resolution and, because of pressure limitations, can be quite slow (1, 2).

In 1976, Chang et al. (3) introduced high-pressure ion-exchange chromatography (HPIEC) of proteins using microparticulate spherical silica. Silica supports are rigid and capable of withstanding high pressure. However, the silica surface must be chemically

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modified for HPIEC to minimize non-specific interactions between mobile solutes (e.g., proteins) and the stationary phase matrix.

Mixed-mode behavior occurs in HPIEC when, in addition to the primary interaction (i.e. ion-exchange), a secondary separation mechanism (e.g. hydrophobic) is found (4). Although a mixed-mode column as such may offer unique selectivities for protein separations, its major problems are poor protein recovery, tendency to denature protein, and less predictable chromatographic behavior (2, 5, 6). Most high-performance liquid chromatography (HPLC) columns available today for protein separation show various degrees of mixed-mode behavior1. The most common mixed-mode in HPIEC reported is the reversed phase (hydrophobic) mode (4, 5, 7, 8).

Hydrophobic mixed-mode behavior can be minimized by adding organic modifier to mobile phase (e.g., 10% isopropanol); maximizing the hydrophilicity of the stationary phase provides an alternative approach. Here, we report the coupling of glucuronic acid, a hydrophilic ionic molecule, to aminopropyl derivatized silica. Potential uses of this glucuronic acid-silica as a cation-exchange HPLC support were investigated.

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METHODS

Chromatography: The Chromatograph was a Rainin Rabbit-HP solvent delivery system outfitted with a Knauer variable wavelength detector, a Macintosh SE computer, and Rainin Dynamax version 1.2 software for data collection and analysis. Chromatography was at room temperature (20 ^{o}C) with a 1 ml/min flow-rate throughout.

Preparation of Glucuronic Acid-silica: Glucuronic acid-silica was prepared by a modification of the procedure used to couple glucose to silica (9). Five milliliters (1 mmol) 0.2 M D-glucuronic acid (Aldrich Chemical Company, Inc., Milwaukee, Wis.), 0.4 M sodium phosphate, pH 6.8, and 126 mg (2 mmol) $NaCNBH_3$ was reacted per gram of 7-µm aminopropyl-silica (300 Å pore) at 60 ^{o}C for 5 h. The reaction was carried out twice in order to obtain product which was consistently Cd-ninhydrin10 negative at room temperature. Glucuronic acid-silica after the first reaction was washed with water only and after the second reaction was first washed with acetone and then with water. The product was then dried from acetone at 60 ^{o}C.

Titration of Glucuronic Acid-silica: Glucuronic acid (100 mg) was titrated in 10 ml 1 M NaCl. Enough 0.1 M HCl was initially added to bring the pH to 2. Then 50 µl portions of 0.1 M NaOH were added and the pH recorded until it reached 12. pH was monitored

using a Corning pH Meter Model 220 and Orion Combination pH Electrode (Model 91-02).

Hemoglobin (Hb) Binding Capacity Determination: Glucuronic acid-silica and glucose-silica (50 mg each) were equilibrated in 20 mM acetic acid-NaOH, pH 5. The supports were then mixed with 1 ml of 15 mg/ml Hb for 15 minutes. The amount of Hb bound was determined spectrophotometrically by measuring the absorption of the supernatant at 410 nm, based on one absorbance unit = ~0.158 mg/ml Hb (11). Glucose-silica, which does not bind Hb, was used as control.

Elemental Analysis: Glucuronic acid-silica and aminopropyl-silica were sent to Galbraith Laboratories, Inc. (Knoxville, TN) for elemental (C, N, and H) analysis.

Column Packing: Glucuronic acid-silica was packed into 100 x 4.6 mm I.D. columns using a stainless steel reservoir and a Rainin Chromatography pump. A slurry containing 2 grams glucuronic acid-silica and 4 ml water was prepared to fill the column and reservoir. Using water as the solvent, the flow-rate was initially at 0 ml/min at time zero and increased linearly to 5 ml/min in five minutes, remained at 5 ml/min for next ten minutes, and returned back to 0 ml/min by 20 minutes.

Column Testing: Columns were tested as described in figure legends. All proteins and biochemicals used for column testing were from Sigma Chemical Co. (St. Louis, MO). Columns were stored at 4 ^{o}C in 10 mM sodium azide, 10 mM acetic acid-NaOH, pH 5.

Hydrophobicity Test: Concentrated lysine and lysyl-leucine (> 10 mg/ml) were prepared in aqueous solution. Each was injected in triplicate on the column. Both separations used a 10 minute linear gradient from 0 M NaCl to 0.1 M NaCl in 10 mM acetic acid-NaOH, pH 5. Detection was at 230 nm.

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RESULTS

The titration of glucuronic acid-silica is shown in Fig. 1. The only titratable groups observed have a pKa in the pH 3-4 range. The carboxylic acid at carbon six of a series of glucuronides also has a pKa in this range (12, 13). This low pKa should allow the support to be used for chromatography over a larger pH range than the carboxymethyl type cation-exchangers (pKa ~4.7).

The protein binding capacity of glucuronic acid-silica was quite high. Glucuronic acid-silica bound 202 mg-hemoglobin per gram at pH 5. In agreement with a pKa in the

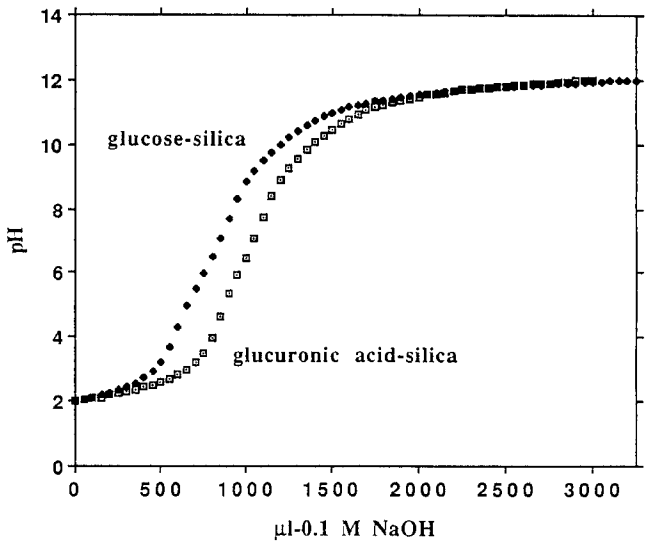

FIGURE 1. Titration curves of glucuronic acid-silica and glucose-silica.

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pH 3-4 range, about 10% as much hemoglobin bound at pH 3 and almost none bound at pH 2.5. The shift in the titration curve for glucuronic acid-silica relative to a glucose-silica control (Fig. 1) gave a measure of 0.2 milliequivalents per gram for this particular batch of glucuronic acid-silica. On a different batch of material, we found by elemental analysis that the support contained 0.11 mmol glucuronic acid per gram silica. Thus, capacities in the range of 0.1-0.2 milliequivalents per gram were found.

All of the glucuronic acid-silica prepared as described gave no detectable reaction with the Cd-ninhydrin reagent at room temperature suggesting that no unreacted aminopropyl groups remain. However, elemental analysis revealed this is not the case. Elemental analysis of a typical batch of support gave 0.11 mmol glucuronic acid and 0.38 mmol aminopropyl per gram silica showing that reaction was only about 30% complete. Although the reaction used to synthesize glucuronic acid-silica was carried out twice, steric hindrance and charge repulsions between adjacent glucuronic acids might account for this observed low coupling efficiency. Why Cd-ninhydrin reaction failed to detect remaining aminopropyl groups is unclear but may suggest that the reagent is sterically excluded from the aminopropyl layer. However, the pH dependency

of hemoglobin binding already discussed suggests that glucuronic acid-silica is a high capacity cation-exchanger which was next characterized chromatographically.

In Fig. 2, four proteins (carbonic anhydrase, myoglobin, cytochrome c, and lysozyme) were found to elute in the order predicted from their pI values (5.9, 6.9, 9.4, and 11.0, respectively). The unlabeled peak eluting near six minutes is a contaminant found in the cytochrome c used. Proteins with basic isoelectric pH (pI) values such as lysozyme and cytochrome c should bind tighter to the column and be eluted later than proteins with more neutral pI values such as carbonic anhydrase and myoglobin. Although this relationship was demonstrated by the separation of the proteins selected, injection with bovine pancreas ribonuclease A (pI 8.7) (14) disobeyed the relationship. It eluted before myoglobin while it should elute after based upon pI (data not shown). Kopaciewicz et al. (15) have shown that the chromatographic behavior of proteins is not entirely predictable based upon pI value alone and have suggested this is due to the complexity of protein structure. Alternatively, our results with ribonuclease A may be due to mixed chromatographic modes due to residual, unreacted propylamines.

Protein recovery was high as shown in Fig. 3. Five injections of myoglobin were eluted with an average recovery of 95%. The fluctuation seen was probably due to combined errors in injections and protein concentration determinations. In general, high dynamic protein mass recovery is indicative of a hydrophilic column since hydrophobic mixed-mode columns generally give poor protein mass recovery (5, 6). Hydroxyl groups provided by glucuronic acid on the silica surface should contribute significantly to this hydrophilicity.

A hydrophobicity test was designed to investigate more directly if glucuronic acid-silica also had hydrophobic interactions with applied samples. Two compounds of equal charge but different hydrophobicity were used to assess hydrophobic interaction with the stationary phase. The compounds were lysine (lys) and lysyl-leucine (lys-leu) with the latter being the more hydrophobic. Results summarized in Table I show lys-leu was retained significantly longer than lys by the column under the same conditions. A column without any hydrophobic interaction should give the same retention times for both compounds. The results suggest that glucuronic acid-silica also has some hydrophobic interaction with these samples though the effect is relatively minor.

Fig. 4 shows the results of a dynamic stability test performed on glucuronic acid-silica. Lysozyme, myoglobin, benzylamine, and phenylacetic acid were examined for five 8 h. days of rigorous chromatography. The results show that retention times of both proteins were slightly decreased throughout. The biggest change, however, occurred

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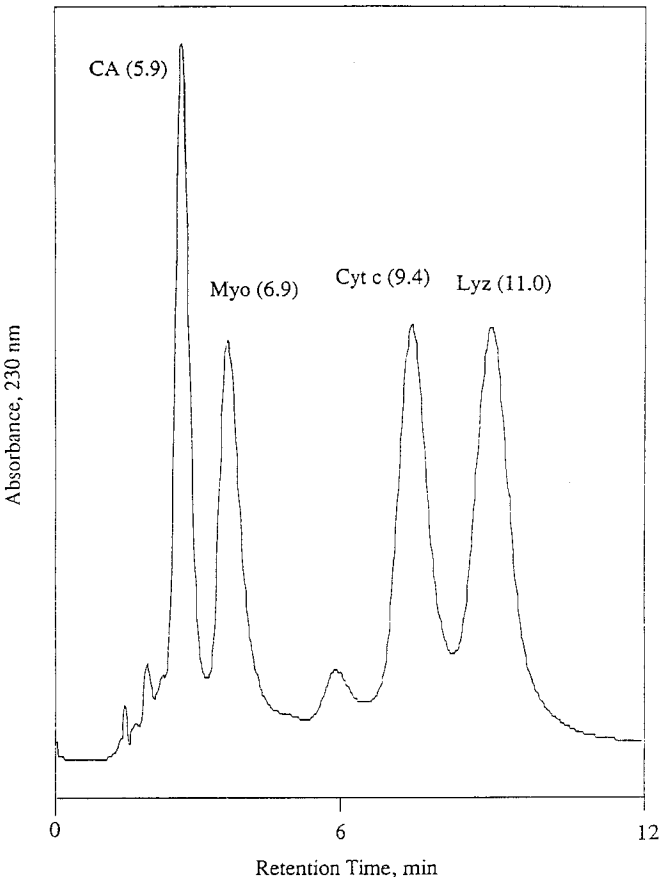

FIGURE 2. The cation exchange chromatography separation of four proteins is shown. A protein mixture containing bovine carbonic anhydrase (CA), horse heart myoglobin (Myo), horse heart cytochrome c (Cyt c), and chicken egg white lysozyme (Lyz) were separated in order of their isoelectric pH values shown (on the figure in parentheses) (14, 15). Buffer A (20 mM acetic-NaOH, pH 5) and buffer B (0.5 M NaCl in buffer A) were used in a 10 minutes linear gradient from 30% to 40% buffer B. Injection was 10 µl of ca. 4 mg/ml protein mixture. Each protein was also injected individually onto the column to confirm the identity of each peak in the mixture.

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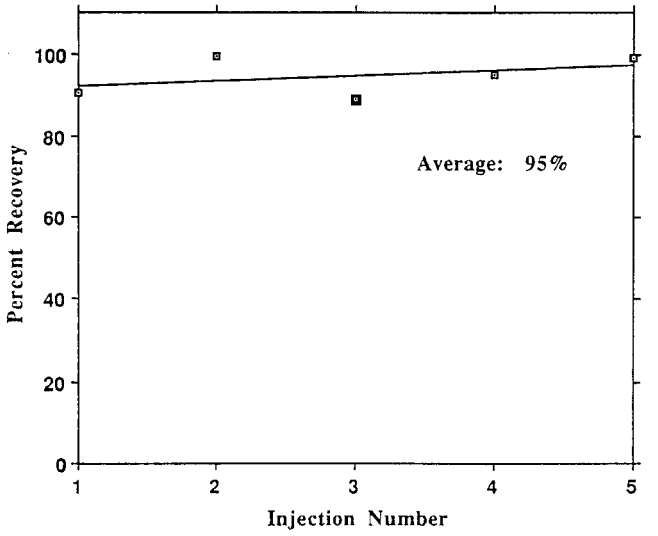

FIGURE 3. Recovery of myoglobin injected onto a glucuronic acid-silica column. After two test runs, the glucuronic acid-silica column was used to study the dynamics of protein recovery. Each injection was 10 µl of 10 mg/ml myoglobin. The bound myoglobin was eluted from the column using a 10 minute linear gradient from buffer A (20 mM acetic acid-NaOH, pH 5) to buffer B (20 mM acetic acid-NaOH, 1 M NaCl, pH 5). Peak fraction was collected and the myoglobin concentration was determined16 using bovine serum albumin as standard. 100% recovery is that found when a zero dead volume union was used in place of the column.

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TABLE 1.

Elution* (Column Volumes)		
Lysine	Lysyl-leucine	% Difference
1.506 ± 0.055	1.662 ± 0.020	9.4 ± 4.4

*Retention times are expressed as the mean ± the standard deviation with n = 4.

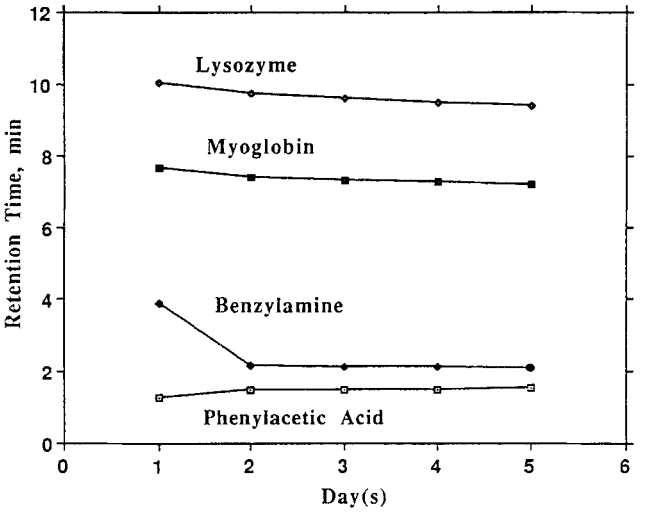

FIGURE 4. Dynamic stability of glucuronic acid-silica. A glucuronic acid-silica column was subjected to continuous repetitions of a chromatographic cycle (15 minute linear gradient from 0 M to 0.5 M NaCl in 20 mM acetic acid-NaOH, pH 5, followed by a 10 minute reequilibration with the zero salt buffer, then repeat) for 8 hours each day except the first day. The first day was devoted to working out the method, obtaining the initial chromatograms, and about four hours of repetitive chromatography. Subsequent chromatograms were obtained each day in the morning and evening and the retention times for these two runs were averaged. Average retention times for each day are plotted.

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with benzylamine between day 1 and day 2. The results suggest that the anionic characteristic of glucuronic acid-silica diminished during the initial day of chromatography but remained fairly constant afterward. Behavior of oppositely charged phenylacetic acid whose retention time was increased by the second day also agrees with this conclusion. One likely explanation for the phenomenon is that some portion of the glucuronic acids is attached noncovalently and washed away during initial hours of chromatography. Despite the behavior observed with benzylamine and phenylacetic acid, the column gives reasonably constant retention of the proteins and gave quite consistent performance for both proteins and small molecules after an initial one day washing period.

The long term stability of glucuronic acid-silica was also investigated over a period of two years and nine months. When not in use, the column was stored at 4^0 in buffer A containing 10 mM NaN_3. At the end of the period, the separation in Fig. 2 was repeated and again all four proteins were well separated and eluted in the same order although column performance was somewhat degraded (data not shown).

DISCUSSION

A novel and simple way of making HPLC cation-exchange silica-based support is described. The support has a lower pKa than the conventional carboxymethyl type and high Hb static binding capacity at pH 5. Despite the presence of residual cationic propylamine moiety, four basic proteins were easily separable under the conditions used and overall our study suggests that glucuronic acid-silica is a high-performance cation-exchanger. It also exhibited relatively minor hydrophobic interactions. The columns also gave high dynamic protein mass recovery and appeared to be fairly stable for protein separations during the time tested. In short, glucuronic acid-silica represents a novel hydrophilic support suitable for high-pressure cation-exchange chromatography of proteins.

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ACKNOWLEDGEMENT

This work was supported by NIH (GM43609). The excellent technical assistance of Larry R. Massom and Timothy Luong is appreciated.

REFERENCES

1. H.H. Freiser and K.M. Gooding, J. Chromatogr., 544: 125-135 (1991).
2. M.P. Henry, J. Chromatogr., 544: 413-443 (1991).
3. S.H. Chang, K.M. Gooding, and F.E. Regnier, J. Chromatogr., 120: 321-333 (1976).
4. M.L. Heinitz, L. Kennedy, W. Kopaciewicz, and F.E. Regnier, J. Chromatogr., 443: 173-182 (1988).
5. W. Kopaciewicz, M.A. Rounds, and F.E. Regnier, J. Chromatogr., 318: 157-172 (1985).
6. T.W. Hearn, F.E. Regnier, and C.T. Wehr, American Laboratory, Oct., 1982, pp. 18-39.

7. L.A. Kennedy, W. Kopaciewicz, and F.E. Regnier, J. Chromatogr., 359: 78-84 (1986).
8. K.M. Gooding and M.N. Schmuck, J. Chromatogr., 327: 139-146 (1985).
9. H.G. Lee and H.W. Jarrett, J. Chromatogr., 511: 69-78 (1990).
10. W.J. Dryer and E. Bynum, Methods Enzymol., 11: 32-39 (1967).
11. M.A. Rounds, W.D. Rounds, and F.E. Regnier, J. Chromatogr., 397: 25-38 (1987).
12. A.J. Quick, J. Biol. Chem., 97: 403-419 (1932).
13. D. Robinson, J.N. Smith, and R.T. Williams, Biochem . J., 53: xxvii (1953).
14. G.L. Righetti and T. Caravaggio, J. Chromatogr., 127: 1-28 (1976).
15. W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, and F.E. Regnier, J. Chromatogr., 266: 3-21 (1983).
16. M.M. Bradford, Anal. Biochem., 72: 248-254 (1976).

Received: August 29, 1994
Accepted: October 18, 1994

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