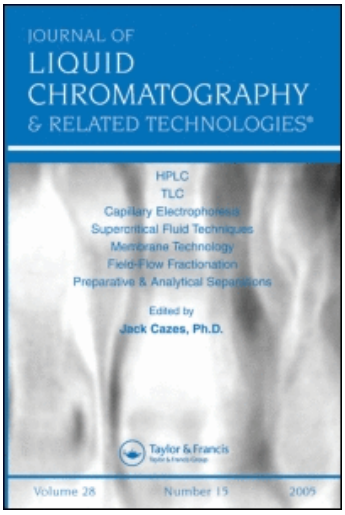

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STUDIES OF THE INFLUENCE OF HYDROGEN CHLORIDE ON THE LIQUID CHROMATOGRAPHIC PROPERTIES OF SILICA-IMMOBILIZED BOVINE SERUM ALBUMIN UNDER NORMAL-PHASE CONDITIONS

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ABSTRACT

The influence of hydrogen chloride on the retention properties of silica-immobilized bovine serum albumin has been studied using a series of simple aromatic phenols and acids. The result of treating the protein with HCl is to mediate hydrogen bonding interactions between the solutes and basic sites in the protein or unreacted amino groups on the surface. The current observations are consistent with previously reported work which examined the influence of HCl on aminopropyl surfaces.

INTRODUCTION

Bovine serum albumin (BSA) is a large flexible "cigar-shaped" protein with a molecular weight of 66,267. The protein has the natural ability to selectively bind a number of ligands including enantiomers such as L-tryptophan and related L-analogs, whereas the corresponding D-isomers do not.[1] A number of investigators have used the silica-immobilized BSA as a

chiral selective stationary phase in liquid chromatography to resolve racemic compounds.[2-12] The separations are carried out in combination with aqueous buffered eluents to insure that the protein retains much of its native conformation and indigenous binding properties. However, to date, little has been done to investigate silica-immobilized BSA as a normal-phase liquid chromatographic packing.[13,14]

Because proteins are extremely complex biomolecules with tertiary structures stabilized by disulfide bridges, as well as numerous intra- and intermolecular hydrogen bonding, the number of possible sites where a polar solute molecule may interact, as well as the heterogeneity of these interactions, is great. In the case of BSA, the protein is made up of 582 amino acids with varying α-substituents folded into three domains. To complicate the situation further, the interactions of BSA with numerous organic and inorganic ligands is well known.[15] The protein will bind both anions (chloride, fluoride, bromide and iodide, etc.) and cations (Cu^{++}, Zn^{++}, Ca^{++}, Mg^{++}, etc.).[2]

Since the possibility of hydrogen bonding between ligands and native BSA is known to be an important factor which influence binding, similar strong interactions should potenitally be useful for controling solute retention under normal-phase eleuent conditions. This approach recently has been used to separate phenolic solutes with a ternary mixture of hydrogen chloride-diethyl ether-hexane eluent.

Recently, Ehtesham and Gilpin,[16] have studied the influence of hydrogen chloride on the retention properties of aminopropyl bonded phases under normal-phase eluent conditions by measuring differences in solute retention before and after converting the surface to its corresponding hydrochloride salt. The result of treating the amino groups with HCl was to dramatically decrease the interactions of hydroxylated solutes. In a similar fashion, the influence of hydrogen chloride on the chromatographic properties of silica-immobilized BSA under normal-phase conditions have been examined. The capacity factors for a series of simple hydroxylated and acid containing aromatic compounds have been studied before and after the protein surface was modified by passing a dry HCl-saturated solution of diethyl ether through the column.

The result of this treatment was to mediate the polar interactions which exist between the bonded BSA surface and polar solutes. Thus, solutes which were strongly retained by the unmodified protein could be eluted in a significantly shorter time.

EXPERIMENTAL

Chemicals

All compounds used as solutes as well as the 25% solution of glutaric dialdehyde, the ethanol (anhydrous and denatured with 5% 2-propanol) and the hydrogen chloride (220 g lecture cylinder) were purchased from the Aldrich Chemical Company (Milwaukee, WI, USA). The anhydrous diethyl ether and the HPLC grade 2-propanol, methanol and hexane were from the Fisher Scientific Company (Pittsburgh, PA, USA), the bovine serum albumin (fatty acid free), sodium phosphate (reagent grade) and sodium cyanoborohydride were from Sigma (St. Louis, MO, USA), and the 3-aminopropyltriethoxysilane was from Huls (Bristol, PA, USA). The deionized water was produced in-house using a Millipore (El Paso, TX, USA) model Milli-Q purification system.

Instrumentation

The chromatographic system consisted of a Spectra-Physics (San Jose, CA, USA) model SP8810 precision isocratic pump, model SpectroMonitor III variable wavelength UV detector, and model 4400 Datajet integrator. The samples were introduced using a Rheodyne (Berkeley, CA, USA) model 7125 injection valve with a 20μL loop. In order to insure controlled eluent conditions, the flow rate of the mobile phase was monitored with a Phase Separation LTD (Queensberry, Clwyd, UK) model FLOSOA1 flow meter and the column temperature was controlled in a water bath equipped with a Fisher Scientific model 730 isotemp immersion circulator and a Neslab Instrument (Portsmouth, NH, USA) model EN-350 flow-through cooler.

Column Preparation

The silica-immobilized BSA was prepared as follows. Approximately 2.3 g of Licrospher Si-300 silica (EM Separations, Gibbstown, NJ, USA) were washed with 25 mL of deionized water and the water was decanted off after centrifugation. The silica was dried at 85°C for 4 hours and transferred to a glass reaction vessel. Subsequently, 10 mL of a 10% (v/v) aqueous solution of 3-aminopropyltriethoxysilane were added and the pH of the mixture was adjusted to 3.4 via addition of phosphoric acid. After allowing the reaction to proceed for 3 hours at 75°C, the aminopropyl-silica was washed using six portions of 25 mL deionized water (i.e., the material was centrifuged and the water decanted off between each wash). The product was dried at 100°C

overnight and a small portion of the material was removed for elemental analysis. The remainder (2.4 g) of the modified silica was placed in a flask and 100 mL of a 2.5% aqueous glutaric dialdehyde (10 mL of 25% glutaric dialdehyde and 90 mL 0.05M pH 7 phosphate buffer) and 0.13 g of sodium cyanoborohydride were added. The mixture was allowed to stand for 3 hours after which time the aldehyde activated silica was washed with five 30 mL portions of deionized water and the material freeze dried.

The final protein coupling step was carried out by the drop wise addition of bovine serum albumin (30 mL of 1% BSA diluted with 55 mL of 0.05M pH 7 phosphate buffer) to a flask which contained 2.0 g of the aldehyde-activated silica, 85 mL of 0.05M pH 7 phosphate buffer and 0.1 g sodium cyanoborohydride. The reaction was allowed to proceed 0.5 hour, another 0.1 g of sodium cyanoborohydride was added, and the reaction continued for 12 hours. The resulting silica-immobilized BSA was then washed with 150 mL of deionized water and freeze dried.

A sample of this material was analyzed by Huffman Laboratories (Golden, CO, USA) for carbon, nitrogen and sulfur. The resulting data indicated a coverage of 67 mg of immobilized protein/g of silica.

Approximately 0.5 g of the dried silica-immobilized BSA was added gradually to 30 mL of denatured anhydrous ethanol which was contained in a dynamic packing apparatus. The apparatus was sealed and pressurized to 6000 psi using a Haskel (Burbank, CA, USA) model DST-52 air driven fluid pump with ethanol as the carrier solvent. During this process the 2.1 mm i.d. X 150 mm stainless column were backed in upward fashion.

Chromatographic Studies

The compounds used as test solutes were dissolved in a 17% solution of 2-propanol in hexane at a concentration of ~ 1 mg/mL. Retention data were collected using mobile phases of 100% hexane, 5:95 2-propanol-hexane (v/v) and 10:90 2-propanol-hexane (v/v).

These experiments were carried out on the silica-immobilized BSA before and after it had been treated with a hydrogen chloride-saturated diethyl ether solution. In order to determine the completion of this latter treatment, aliquots were collected at 5 mL intervals, each of these mixed with 25 mL of distilled water and the pH of the water layer measured.

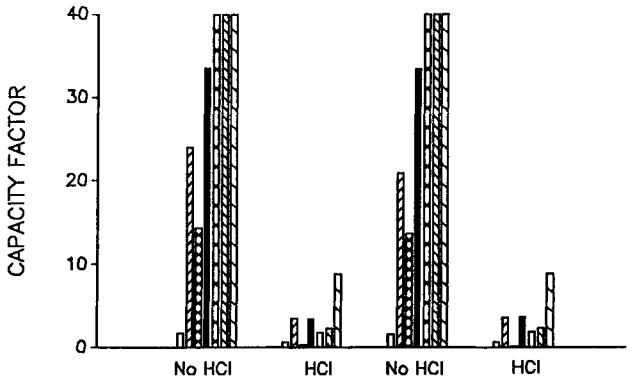

Figure 1. Histogram showing the effect of hydrogen chloride on the retention of the silica-immobilized BSA phase. Mobile Phase: 10% 2-propanol in hexane; Solutes: Graphed in the order presented in Table 2. Note: Bars ending at 40 denote a k' of at least 40 or more. Toluene not shown because k' values are too small to appear on the scale.

RESULTS AND DISCUSSION

Retention measurements were carried out on silica-immobilized BSA before and after treatment with hydrogen chloride. A total of seven sets of retention measurements were made on two different columns cycling between BSA and BSA-HCl. Between each cycle the column was washed with approximately 400 mL of methanol to remove the HCl. As discussed below, this washing was sufficient to remove most of the HCl, but not all, which resulted in incomplete recovery of the columns. In making these measurements, a total of eight organic compounds were selected as test solutes which ranged from a simple aromatic, toluene, to aromatic compounds which contained either one or more polar hydroxyl or carboxyl groups.

An initial set of scouting measurements were made in order to establish eluent conditions of sufficient strength that a majority of the test solutes would elute within one hour. This was done using column 1. A total of three different mobile phases were tried starting with 100% hexane, then hexane with 5% 2-propanol as the polar modifier and finally hexane with 10% 2-propanol. Next, the column was converted to its HCl form under nonaqueous conditions and the measurements repeated for the three mobile phases. The results of this scouting experiment are summarized in Table 1.

Table 1

Retention Data for Silica-Immobilized BSA Before and After Treatment with HCl

	Without HCl	With HCl
Mobile Phase: 10% 2-Propanol in Hexane		
Toluene	0.05	0.03
Phenol	1.57	0.62
Resorcinol	20.9	3.58
Benzoic Acid	13.7	0.20
Methyl 3,5-dihydroxybenzoate	33.4	3.76
3-Hydroxybenozic Acid	Ret.	1.89
4-Hydroxybenzoic Acid	----	2.36
3,5-Dihydroxybenzoic Acid	----	8.86
Mobile Phase: 5% 2-Propanol in Hexane		
Toluene	0.04	0.03
Phenol	3.54	0.70
Resorcinol	99.1	6.96
Benzoic Acid	23.3	0.32
Methyl 3,5-dihydroxybenzoate	Ret.	7.07
3-Hydroxybenzoic Acid	----	3.27
4-Hydroxybenzoic Acid	----	5.10
3,5-Dihydroxybenzoic Acid	----	28.1
Mobile Phase: 100% Hexane		
Toluene	0.08	0.07
Phenol	Ret.	25.4
Resorcinol	----	Ret.
Benzoic Acid	----	Ret

Subsequently, additional experiments were carried out on the same column as well as on a second duplicate column cycling between the BSA and BSA-HCl forms of the surface using hexane with 10% 2-propanol as the eluent. The results from this latter study are given in Table 2.

Table 2

Retention Data Illustrating the Reproducibility of HCl Cycling

		Without HCl	With HCl
Toluene	cycle 1	0.04 ± 0.01	0.03 ± 0.01
	cycle 2	0.05 ± 0.00	0.03 ± 0.00
Phenol	cycle 1	1.57 ± 0.15	0.59 ± 0.04
	cycle 2	1.42 ± 0.18	0.62 ± 0.01
Resorcinol	cycle 1	21.2 ± 3.1	3.49 ± 0.04
	cycle 2	18.8 ± 2.5	3.58 ± 0.06
Benzoic Acid	cycle 1	12.3 ± 3.5	0.27 ± 0.04
	cycle 2	11.1 ± 3.1	0.20 ± 0.07
Methyl 3,5-dihydroxybenzoate	cycle 1	34.8 ± 1.4	3.54 ± 0.00
	cycle 2	28.8 ± 5.3	3.76 ± 0.01
3-Hydroxybenzoic Acid	cycle 1	Ret.*	2.83 ± 0.01
	cycle 2	Ret.	1.89 ± 0.00
4-Hydroxybenzoic Acid	cycle 1	Ret.	2.26 ± 0.03
	cycle 2	Ret.	2.36 ± 0.01
3,5-Dihydroxybenzoic Acid	cycle 1	Ret.	10.7 ± 2.1
	cycle 2	Ret.	8.86 ± 0.06

* Retained with a capacity factor greater than 40.

The variation in capacity factor values obtained between the duplicate columns were within 3-5% for the unmodified BSA and better than 2% for the HCl treated protein. However, comparing the results between the untreated and HCl treated columns, there was a large reduction in retention for the solutes which interact with basic sites. This effect was enhanced as multiple substituents were added to the solute. To further illustrate this dramatic effect the average of the two columns through two complete cycles are presented as a histogram in Figure 1.

The current results are consistent with previously observed reductions in retention for similar hydroxylated solute on aminopropyl bonded phases following conversion of the surface to its hydrochloride salt.[16] The effect of the HCl treatment is to mediate the strong interactions which can occur between the solutes and the basic sites in the protein, as well as possible unreacted basic sites on the surface, since the protein is coupled to the surface via aminopropyl groups. A second feature of the current work, was to illustrate that the protein can be reversibly altered by treatment with HCl as long as eluent conditions are dry. Although there appears to be a small reduction in retention comparing the date from cycle 1 and cycle 2 for both columns (i.e., the retention was always slightly longer in cycle 1 than cycle 2), statistically the results are equivalent within 1 standard deviation unit. The slight reduction, if real, is probably due to trace amounts of HCl retained on the column, even after washing with 400 mL of methanol.

CONCLUSIONS

The strong interactions between silica-immobilized BSA and solutes that contain polar functionality such as hydroxyl and carboxyl groups can be decreased by converting the protein to its HCl form. Such an approach (i.e., the use of protein modifiers) may be useful to create a variety of novel chromatographic supports. It appears that the modification of BSA is reversible assuming that completely anhydrous conditions are maintained. Further experiments are in progress to characterize the normal-phase behavior of silica-immobilized BSA and BSA-HCl, as well as other protein modifiers.

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