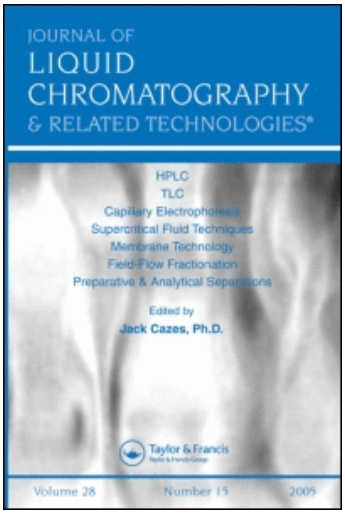

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TYPE C AMINO COLUMNS FOR AFFINITY AND AQUEOUS NORMAL PHASE CHROMATOGRAPHY: SYNTHESIS AND HPLC EVALUATION

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TYPE C AMINO COLUMNS FOR AFFINITY AND AQUEOUS NORMAL PHASE CHROMATOGRAPHY: SYNTHESIS AND HPLC EVALUATION

Maria T. Matyska, Joseph J. Pesek, and Geeta Shety
San Jose State University, San Jose, California, USA

□ *The synthesis of novel stationary phases for the retention of polar compounds and for affinity chromatography was presented. Two amino compounds, 4-diethylamino-2-butyn-1-ol and 3-amino-3-methyl-1-butyne, were bonded to a silica hydride surface using Silica-C and a hydrosilation procedure. The bonding was confirmed using CP – MAS NMR and FTIR techniques. High Performance Liquid Chromatography (HPLC) evaluation of the synthesized HPLC stationary phases was performed using the Neue Test. Aqueous Normal Phase (ANP) properties of synthesized amino phases were confirmed using a broad range of polar compounds. Type III-O trypsin inhibitor from chicken egg white was used to test the affinity aspect of the amino bonded trypsin column.*

Keywords affinity chromatography, amino stationary phases, aqueous normal phase (ANP), retention of polar compounds, type-C silica columns

INTRODUCTION

High performance affinity chromatography (HPAC) relies on the basic principle of HPLC and the interaction of biological molecules to its substrates. In this technique, a specific binding agent is used for the separation of the desired target analyte. Due to the highly specific nature of the stationary phase, it becomes easier to purify a specific analyte from a complex mixture without interference from other sample components.[1] An immobilized agent, known as the "affinity ligand" is covalently attached to the stationary phase. The affinity ligand can be one of the two interacting pairs and the target molecule is analyzed by injection onto the column using

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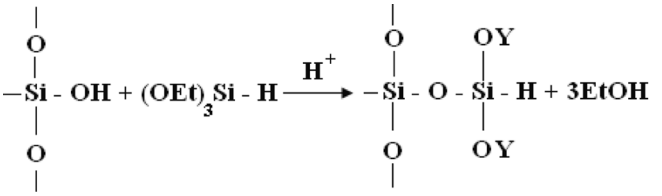
traditional HPLC methods. There are numerous manuscripts describing use of affinity columns in many aspects of separation science.[2–5]

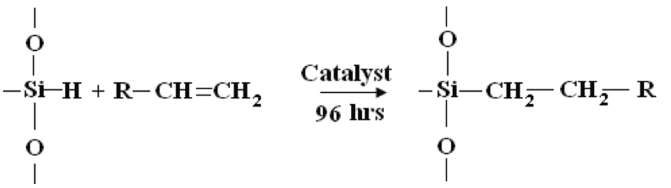
In this manuscript, a technique developed by Pesek, et al.[6] was used to produce new amino stationary phases. The best amino stationary phase synthesized was then used to create an affinity column. Pesek's synthesis procedure is a two step process, where the first step involves the preparation of a silica hydride intermediate and the second step involves the attachment of the desired organic group. In the first step, the silanization reaction, the silanol group (Si−OH) is converted into silica hydride (Si−H) under optimized reaction conditions as shown in Reaction 1, where Y=Si or H. The goal of this reaction is to replace most of the silanol groups with silica hydride in the presence of triethoxysilane, water, an acid catalyst, and a suitable solvent.

Unlike the halide intermediate created in the organometallic reaction, the silica hydride species is hydrolytically stable.[6]



(1)

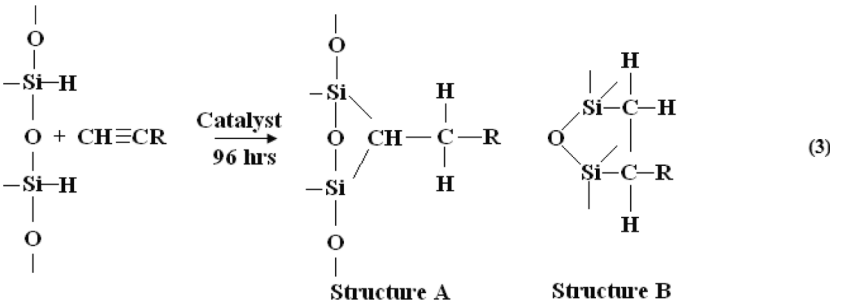
In the second step of this reaction, a terminal olefin is attached to the silica hydride surface through a hydrosilation reaction. Although terminal olefins are the most commonly used for attachment to the hydride surface, other compounds such as alkynes, nitriles, and cyanos can also be bonded in the presence of transition metal complexes. Hexachloroplatinic acid in 2-propanol, also known as Speier's catalyst, is used in this study as the transition metal catalyst as illustrated in Reaction 2. Other organic and inorganic complexes of rhodium, palladium, ruthenium and nickel can also be used as a catalyst for the hydrosilation reaction.[7]



(2)

In this study, two amino compounds, 4-diethylamino-2-butyn-1-ol and 3-amino-3-methyl-1-butyne, are bonded to a silica hydride surface during the hydrosilation procedure. The 4-diethylamino-2-butyn-1-ol bonded phase was synthesized using both platinum and free radical catalysts. The platinum catalyst has a tendency to coordinate with the free amino

group of 3-amino-3-methyl-1-butyne; hence, only a free radical catalyst was used for the bonded phase synthesis of this compound.[8] The NMR studies of this research indicated two possible hydrosilation structures for both the amino compounds as illustrated in Reaction 3.



(3)

In order to get the maximum surface coverage of the bonded phase, an initial synthesis was carried out in small batches to determine optimized conditions followed by big batch synthesis. Once both the amino bonded phases were synthesized, a portion of the 3-amino-3-methyl-1-butyne bonded phase was used to create an affinity column. This column was synthesized by first activating the 3-amino-3-methyl-1-butyne with carbonyldiimadazole followed by attachment of trypsin using a suitable buffer. Once the packing material for all the columns was synthesized, they were chromatographically evaluated using different groups of compounds. The final four columns used for chromatography studies were as follows: 4-amino-4-methyl-1-butyne bonded phase using platinum catalyst, 4-amino-4-methyl-1-butyne bonded phase using free radical catalyst, 3-amino-3-methyl-1-butyne bonded phase using free radical catalyst and 3-amino-3-methyl-1-butyne bonded phase + trypsin (affinity column).

For the initial chromatographic characterization method, the Neue test[9] was done on all four columns. Next, the amino based columns and the protein column were used to analyze a set of sugar samples. Further evaluation of all four columns was done by analyzing metformin and a few tricyclic antidepressants. All the samples were analyzed under aqueous normal phase conditions (ANP). The unique feature of ANP is that the mobile phase is both non-polar and aqueous; this is done by combining small amounts of water with an organic solvent.[10] In ANP chromatography, polar compounds are retained strongly by increasing the amount of organic solvent in the mobile phase. One can also alter the elution order by modifying the amount of water used in the mobile phase. As the amount of water increases in the mobile phase, the polar compounds are eluted faster.[11] The last part of the research involved determining the affinity

aspect of the protein column (3-amino-3-methyl-1-butyne bonded phase + trypsin) using trypsin inhibitor.

EXPERIMENTAL

Materials

Compounds Used for Synthesis

Astrosil™ silica, which has a 4.2 μm average particle size, was used in the synthesis of all the stationary phases. Astrosil™ silica with a specific surface area of 340 m^2/g was purchased from Stellar Phases (Langhorne, PA). All the other compounds used in the synthesis of bonded phases are shown in Table 1.

Solvents and Buffers Used as Mobile Phases

The chromatographic evaluation of stationary phases was done using organic solvents, water, and buffers as mobile phases. For the Neue test, 35% 20 mM K_2HPO_4 / KH_2PO_4 buffer at pH 7 and 65% methanol were used. The sugar analysis was done using different ratios of acetonitrile and water. Metformin and tricyclic compounds were analyzed using different ratios of 20 mM K_2HPO_4/KH_2PO_4 buffer at pH 7 and methanol. Finally, to determine the affinity aspect of the protein column 0.01 M phosphate buffer at pH 7 was used as the application buffer and 0.1% acetic acid was used as the elution buffer. The CAS number and manufacturer of the solvents and buffers used are shown in Table 2.

Samples Analyzed in this Study

Tables 3, 4, 5, and 6 show a list of all the compounds analyzed in this investigation.

TABLE 1 Compounds Used in the Synthesis of Stationary Phases

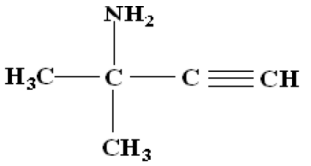
Compound Name	CAS Number	Manufacturing Company
Triethoxysilane	78-07-9	Sigma-Aldrich, St. Louis, MO, USA
Dioxane	123-91-1	Fisher Chemicals, Pittsburgh, PA, USA
Toluene	108-88-3	Fisher Chemicals, Pittsburgh, PA, USA
3-amino-3-methyl-1-butyne	2978-58-7	GFS Chemicals, Columbus, OH, USA
4-diethylamino-2-butyn-1-ol	10575-25-4	Sigma-Aldrich, St. Louis, MO, USA
Diethyl ether	60-29-7	Sigma-Aldrich, St. Louis, MO, USA
Hexachloro platinic acid	16941-12-1	Sigma-Aldrich, St. Louis, MO, USA
t-butyl peroxide	110-05-04	Sigma-Aldrich, St. Louis, MO, USA
1,1′-Carbonyldiimidazole	530-62-1	Sigma-Aldrich, St. Louis, MO, USA
Trypsin	9002-07-7	Sigma-Aldrich, St. Louis, MO, USA

TABLE 2 Compounds Used in the Mobile Phases

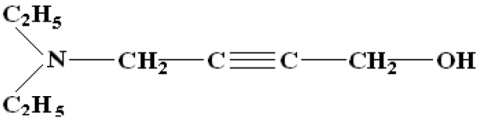
Compound Name	CAS Number	Manufacturing Company
K_2HPO_4	7758-11-4	Fisher Chemicals, Pittsburgh, PA, USA
KH_2PO_4	7778-77-0	J.T. Baker Chemical (Mallinckrodt Baker), Phillipsburg, NJ, USA
Methanol	67-56-1	Fisher Chemicals, Pittsburgh, PA, USA
Acetonitrile	75-05-8	Fisher Chemicals, Pittsburgh, PA, USA
NaH_2PO_4	10049-21-5	Fisher Chemicals, Pittsburgh, PA, USA
Acetic acid	64-19-7	J.T. Baker Chemical (Mallinckrodt Baker), Phillipsburg, NJ, USA

Structures

Amino Compounds Used in the Bonded Phase Synthesis

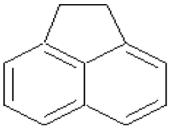


3-amino-3-methyl-1-buytne

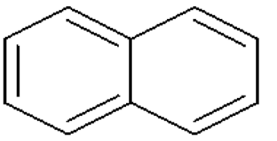


4-diethylamino-2-butyn-1-ol

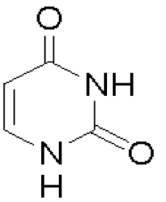
Structures of the Compounds Used in Neue Test



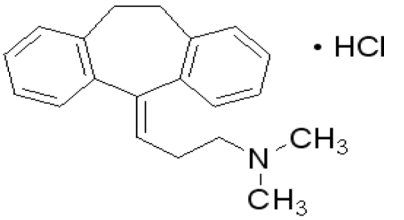
Acenaphthene



Naphthalene



Uracil



Amitriptyline

TABLE 3 List of Samples Used for Neue Test

Compound Name	CAS Number	Manufacturing Company
Uracil	66-22-8	Matheson Coleman & Bell
Naphthalene	91-20-3	Sigma-Aldrich, St. Louis, MO, USA
Acenaphthene	83-32-9	Sigma-Aldrich, St. Louis, MO, USA
Amitriptyline	549-18-8	Sigma-Aldrich, St. Louis, MO, USA

TABLE 4 List of Sugars Used for Chromatographic Analysis

Compound Name	CAS Number	Manufacturing Company
Glucose	50-99-7	Sigma-Aldrich, St. Louis, MO, USA
Sucrose	57-50-01	J.T. Baker Chemical (Mallinckrodt Baker), Phillipsburg, NJ, USA
Fructose	57-48-7	Sigma-Aldrich, St. Louis, MO, USA
Lactose	63-42-3	Sigma-Aldrich, St. Louis, MO, USA
Xylose	58-86-6	J.T. Baker Chemical (Mallinckrodt Baker), Phillipsburg, NJ, USA
Maltose	69-79-4	Fisher Chemicals, Pittsburgh, PA, USA
Raffinose	512-69-6	Fisher Chemicals, Pittsburgh, PA, USA
Melezitose	597-12-6	Sigma-Aldrich, St. Louis, MO, USA

Structures of Sugars Used in Chromatographic Evaluation

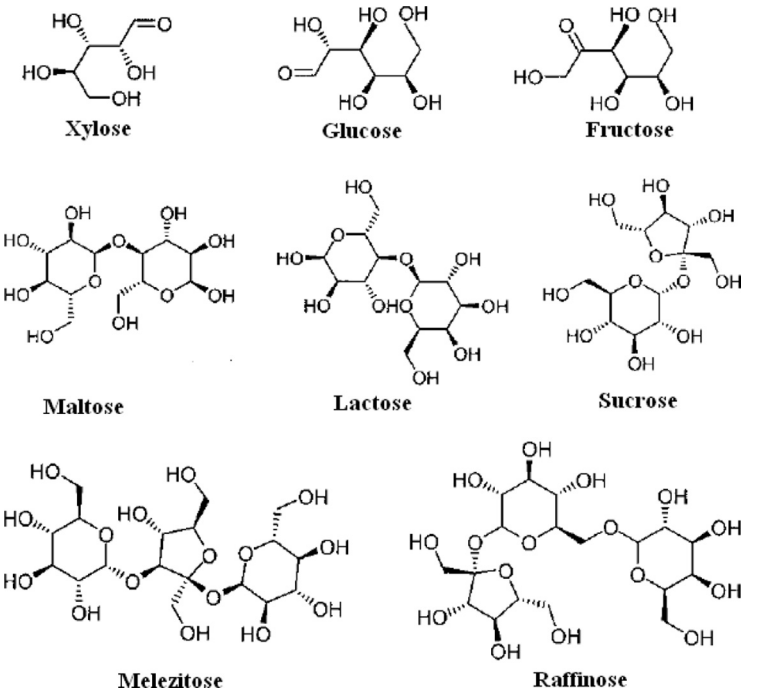


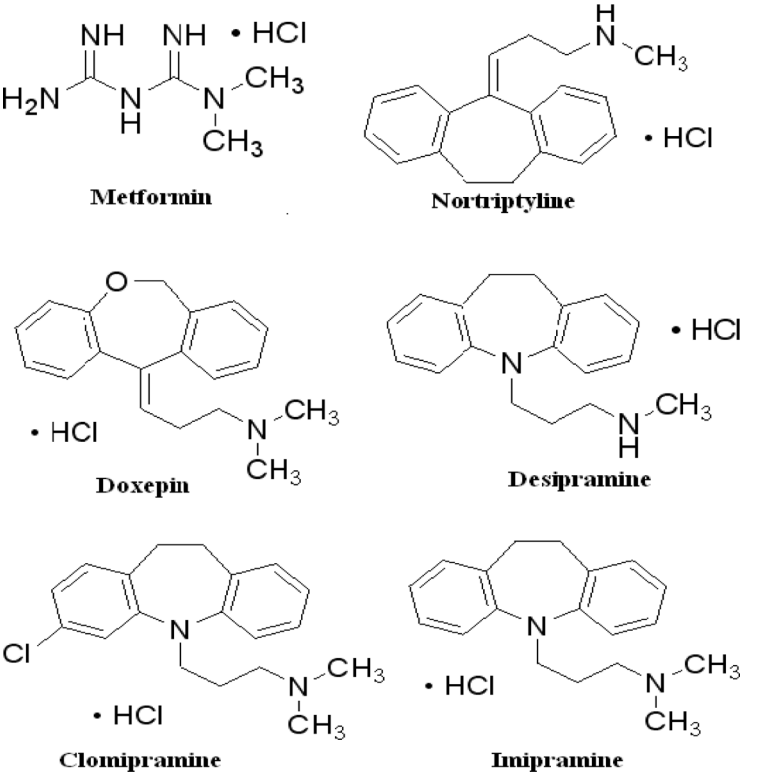
TABLE 5 List of Pharmaceutical Drugs Used in this Study

Compound Name	CAS Number	Manufacturer
Metformin	N/A	Donated to SJSU
Desipramine	58-28-6	Sigma-Aldrich, St. Louis, MO, USA
Doxepin	1229-29-4	Sigma-Aldrich, St. Louis, MO, USA
Clomipramine	17321-77-6	Sigma-Aldrich, St. Louis, MO, USA
Imipramine	113-52-0	Sigma-Aldrich, St. Louis, MO, USA
Nortriptyline	894-71-3	Sigma-Aldrich, St. Louis, MO, USA

TABLE 6 Protein Used in the Study of Affinity Chromatography

Compound Name	CAS Number	Manufacturer
Trypsin inhibitor	9035-81-8	Sigma-Aldrich, St. Louis, MO, USA

Structures of Pharmaceutical Drugs Used in Chromatographic Evaluation



Methods

Synthesis of Silica Hydride

The method has been described previously.[2] Briefly, Astrosil silica (15 g) was weighed out and dried overnight in a vacuum oven at 100°C. After adding the silica, 600 mL of dioxane and 7.29 mL of 2.3 M HCl solution were added to the round bottom flask. This mixture of silica, dioxane, and HCl catalyst was heated to 70°C. Once the reaction temperature was stabilized at 70°C, the mixture of 90.15 mL of dioxane and 20.85 mL of triethoxysilane was added drop-wise to the reaction mixture using an

addition funnel. After the addition of all the TES/dioxane mixture, the temperature was raised to 90°C. The reaction was complete when the entire reaction mixture was heated at 90°C for 90 minutes. The silica hydride formed was then cooled and filtered using vacuum suction. The silica hydride was washed three times each with dioxane, toluene, and diethyl ether. The final product was kept overnight at room temperature to evaporate the ether, followed by drying the silica hydride at 110°C in a vacuum oven for 24 hours. The newly synthesized silica hydride surface was characterized using FTIR.

Synthesis of 4-Diethylamino-2-butyn-1-ol Bonded Phase Using Free Radical Catalyst

Initially, only 0.5 g of silica hydride was used to bond 4-diethylamino-2-butyn-1-ol. The characterization of this bonded phase is discussed later and will be henceforth referred to as small batch synthesis.

For the final stationary phase (big batch synthesis), 3 g of silica hydride was used as the base material. In this reaction, 4.5 mL of 4-diethylamino-2-butyn-1-ol, 60 mL of toluene and 240 μL t-butyl peroxide were used. The reaction mixture was heated at a constant temperature of 70°C +/− 2°C for approximately one hour followed by slow addition of 3 g of silica hydride through the third neck of the flask with constant stirring. Then, the flask was flushed with nitrogen, keeping all the joints properly sealed. After stabilizing the reaction at 70°C, the temperature was raised to 100°C +/− 2°C and the mixture was heated for 96 hrs. The bonded phase which formed was then cooled and filtered using vacuum suction. The bonded phase was washed two times with toluene, followed by diethyl ether. The final product was kept overnight at room temperature to evaporate the ether and was then dried overnight at 110°C in a vacuum oven.[8]

Synthesis of 4-Diethylamino-2-butyn-1-ol Bonded Phase Using Speier's Catalyst

The experimental setup for the bonded phase synthesis of 4-diethylamino-2-butyn-1-ol is similar to the silanization reaction described above. In this hydrosilation reaction, the reaction conditions were optimized by initially creating a small amount of bonded phase followed by big batch synthesis. 0.5 g of silica hydride was used in the small batch synthesis; the characterization results of this bonded phase are discussed later.

For the big batch synthesis, 3 mL of 4-diethylamino-2-butyn-1-ol, 40.02 mL of toluene and 0.78 mL of 10 mM hexachloroplatinic acid in 2-propanol was heated in a 3-necked round bottom flask. The reaction mixture was heated at 70°C +/− 2°C for one hour while being stirred. Then the flask was flushed with nitrogen, keeping all the joints properly sealed. Once the temperature of the reaction mixture was stabilized and a clear

solution obtained, 3 g of silica hydride was added slowly to the third neck of the flask with constant stirring. The reaction temperature was then increased to 100°C +/− 2°C and reaction was continued at this higher temperature for 96 hrs, keeping all the joints sealed. After completion of the reaction, the flask was cooled to room temperature. The newly formed bonded phase was then vacuum filtered and washed three times with toluene and diethyl ether. The final product was kept overnight at room temperature to evaporate the ether and then dried overnight at 110°C in a vacuum oven.[8] The 4-diethylamino-2-butyn-1-ol bonded phase was later characterized using elemental analysis, FTIR, and NMR. The characterization results of these bonded phases are discussed in later sections.

Synthesis of 3-Amino-3-methyl-1-butyne Bonded Phase Using Free Radical Catalyst

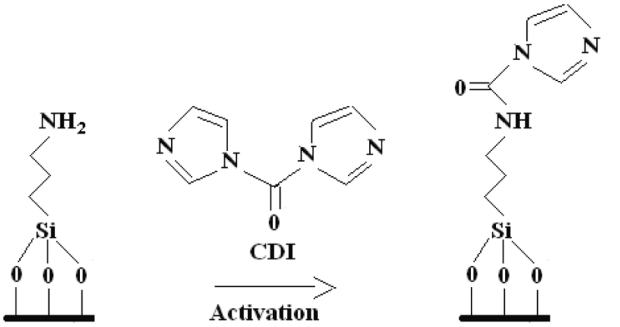
The second amino compound chosen as the bonding moiety for the silica hydride surface was 3-amino-3-methyl-1-butyne. In the small batch synthesis of the 4-diethylamino-2-butyn-1-ol bonded phase, only one reaction condition explained in the previous section was tried. However, for the small batch synthesis of 3-amino-3-methyl-1-butyne bonded phase, it was decided to explore three different reaction conditions. The reaction conditions were optimized by using three different amounts of free radical catalyst in three different set-ups. The experimental set-up in all three small batch syntheses was similar to the silanization reaction described earlier. The different amounts of t-butyl peroxide (free radical catalyst) used in the small batch syntheses were 15 μL, 28 μL, and 45 μL. After careful review of the characterization data, it was decided to use 45 μL of t-butyl peroxide for the big batch synthesis of 3-amino-3-methyl-1-butyne.

The big batch bonded synthesis of 3-amino-3-methyl-1-butyne was carried out according to the standard protocol of the hydrosilation reaction described in the earlier sections. In this experimental setup, 2.7 mL of 3-amino-3-methyl-1-butyne, 84.48 mL of toluene and 270 μL of t-butyl peroxide were heated at 70°C +/− 2°C for approximately an hour. After the addition of 3 g of silica hydride and flushing with nitrogen, the reaction temperature was increased to 100°C +/− 2°C and continued for 96 hrs.[8] The final product was filtered, washed, and vacuum dried according to the same procedure used in previous syntheses, followed by characterization of the bonded phase.

Activation of 3-Amino-3-methyl-1-butyne Bonded Phase for Attachment of Protein

After the hydrosilation reaction of 3-amino-3-methyl-1-butyne was completed, part of the bonded phase was used for the synthesis of the trypsin

bonded protein column. The covalent immobilization of the protein to the bonded stationary phase is a two-step process. The first step involves the activation of the base material and the second step involves the attachment of the desired biological molecule. In this research, the base material used for the activation was the 3-amino-3-methyl-1-butyne bonded phase and the biological molecule attached to the column was trypsin enzyme. The general reaction scheme for this process is shown in Reaction 4.

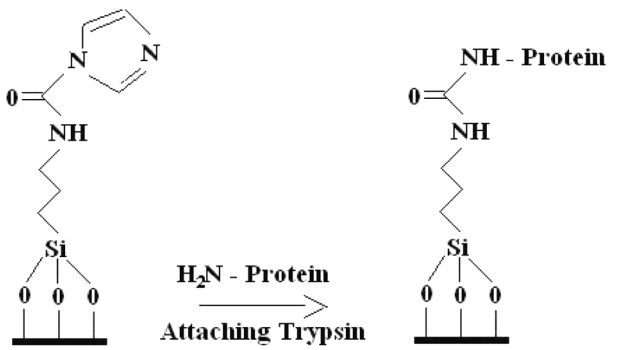


(4)

Prior to the activation process, all the necessary glassware was washed and dried in an oven. The base material, 3-amino-3-methyl-1-butyne bonded silica was dried overnight in the vacuum oven set at 110°C. Following a written protocol,[12] 1.5 g of bonded phase and 2.4 g of 1,1′-carbonyldiimidazole was suspended in 50 mL of dry dioxane. The reaction mixture was stirred for 30 minutes at room temperature. The activated compound was transferred onto a filter crucible. The final product was washed three times with dioxane, acetone and diethyl ether.[12] The activated bonded phase was dried under reduced pressure in a desiccator. The 3-amino-3-methyl-1-butyne activated bonded material was then used for the synthesis of 3-amino-3-methyl-1-butyne + trypsin bonded phase.

Coupling of the Protein to the Activated 3-Amino-3-methyl-1-butyne Bonded Phase

In order to attach the desired protein, it is important to mimic the biological environment of the protein. Hence, instead of using organic solvents for the coupling of protein to the activated bonded phase, 0.1 M phosphate buffer at pH 7 was selected. In this procedure, a suspension of 1.5 g of bonded silica in 75 mL of 0.1 M phosphate buffer and 0.15 g of trypsin was prepared. The reaction mixture was constantly stirred at 4°C for 48 hrs. The final product was transferred onto a filter crucible and washed with 0.1 M phosphate buffer.[12] A general reaction scheme for the process is shown in Reaction 5.



(5)

Trypsin attached to the 3-amino-3-methyl-1-butyne bonded phase was a single polypeptide chain of 223 amino acid residues. A stable linkage between the protein and the activated bonded phase is formed by a nucleophilic substitution reaction between the activated bonded phase and the primary amines on the protein. In this study, a side chain amino group such as arginine and lysine and/or an N-terminal amino group could be responsible for the covalent immobilization of the protein with the amino bonded stationary phase.[13]

Column Packing

After the syntheses of all the stationary phases were completed, they were characterized spectroscopically and packed into stainless steel columns. The four columns were packed in-house with methanol as the driving solvent, using a Haskel pneumatic pump (Burbank, CA). The 4-diethylamio-2-butyn-1-ol bonded phase with platinum catalyst and the 4-diethylamino-2-butyn-1-ol bonded phase with free radical catalyst were packed into 150 mm × 4.6 mm i.d. stainless steel columns. The 3-amino-3-methyl-1-butyne bonded phase with free radical catalyst and the 3-amino-3-methyl-1-butyne + trypsin bonded phase were packed into 100 mm × 4.6 mm i.d. stainless steel columns. Depending on the length of the column, 1.5 g to 2.5 g of bonded phase was suspended into 30 mL of isopropyl alcohol followed by 10 minutes of sonication. This slurry was added to a reservoir tube and the rest of the tube was filled with HPLC grade methanol. The slurry in the reservoir tube was forced into the stainless steel column under high pressure (~6000 psi) in the presence of nitrogen gas. All the stainless steel columns were purchased from Alltech (Deerfield, IL).

Instrumentation

DRIFT spectra were obtained on an ATI Mattson Infinity Series FTIR™ spectrometer equipped with a deuterated triglycine sulfate (DTGS)

detector. The sample was placed in a 3 mm diameter and 2 mm depth diffuse reflectance accessory. The sample for analysis was prepared by mixing 5 mg of KBr with 95 mg of sample; this mixture was dried overnight in a vacuum oven at 110°C. The dried sample was then finely ground with a mortar and pestle, and transferred into the diffuse reflectance cup. In order to obtain a smooth surface, the sample was pressed with a slide and the sample scan was referenced to KBr. Before the analysis, the sample compartment was purged with nitrogen gas for 20 minutes to remove any atmospheric CO_2 and water. The spectra were recorded in the infrared region of 4000 cm^{-1} to 400 cm^{-1}. The spectra were collected at 4 cm^{-1} resolution and averaged over 150 scans.

Elemental Analysis

Elemental analysis was done by Desert Analytics (Tucson, AZ). Equation 6 shows the parameters used in the calculation of surface coverage.

$$\alpha(\mu mole/m^2) = 10^6 P_c/(10^2 M_c n_c\text{-}P_c M_R) S_{BET} \quad (6)$$

In the above equation, P_c is the carbon percentage difference between the bonded phase and the silica hydride; M_c and M_R represent the atomic weight of the carbon atom and the molecular weight of the bonded organic group. BET is the nitrogen absorption method used to determine the surface area of silica and n_c is the number of carbon atoms present in the bonded phase. S_{BET} is the specific surface area of the silica matrix, which is provided by the manufacturer.

Nuclear Magnetic Resonance (NMR) Spectroscopy

In this study, a Varian NMR Inova 400 spectrometer was used with a spinning rate of 5000 Hz. 150 mg of a solid sample (bonded phase) was packed into a double bearing ZrO_2 rotor and the spectra were obtained with a CP contact time of 5 ms. The spectra were analyzed with VNMR 6.1B software.

High Performance Liquid Chromatography (HPLC)

The HPLC instrument used for the analysis of sugars consisted a SP8800 ternary pump, a Chromjet integrator, a laser light scattering detector and a Rheodyne manual injector. The laser light scattering drift tube temperature was set at 63°C. The detection cell temperature was set at 60°C and the pressure of the driving CO_2 was set at 1.25 kg/cm^2. The flow rate of the mobile phase was set at 0.35 mL/min and the pump pressure was set at a maximum of 2000 psi. The chart speed and the integrator attenuation were set at 0.25 cm/min and 64, respectively. The sugar samples were

TABLE 7 Surface Coverage for Small Batch Syntheses

Compound Name	Type of Catalyst	Carbon (%)	Surface Coverage $\alpha = \mu mol/m^2$
3-amino-3-methyl-1-butyne	Free radical (15 μL)	1.65	0.83
3-amino-3-methyl-1-butyne	Free radical (28 μL)	1.76	0.88
3-amino-3-methyl-1-butyne	Free radical (45 μL)	2.76	1.41
4-diethylamino-2-butyn-1-ol	Free radical	8.60	3.02
4-diethylamino-2-butyn-1-ol	Platinum catalyst	9.64	3.44

prepared by dissolving 1.5 mg of sample in 1 mL of 10:90 methanol/water. The Neue test and the analysis of the pharmaceutical drugs were done using Millennium 2.1 software, a Waters 515 HPLC pump, a Waters 717 plus autosampler, and a Waters 991 photodiode array detector. The pharmaceutical drug samples were prepared by dissolving 1 mg of sample in 1 mL of methanol. The mobile phase flow rate was set at 0.5 mL/min. The organic solvents and water used in the mobile phase were degassed using helium gas at a flow rate of 5 mL/min. All four newly packed columns were flushed with methanol for 10 hours before testing them chromatographically.

RESULTS AND DISCUSSION

The surface coverage values for the small batch indicated that both the amino compounds were bonded to the silica hydride surface successfully. However, the 4-diethylamino-2-butyn-1-ol bonded phase had better surface coverage with both Pt catalyst and free radical catalyst compared to the 3-amino-3-methyl-1-butyne bonded phase with free radical catalyst. For the 3-amino-3-methyl-1-butyne bonded phase synthesis, 45 μL of free radical catalyst provided the best surface in comparison to the 15 μL and 28 μL free radical catalyst. Hence, the 45 μL reaction condition was used for the big batch synthesis. The results of the big batch synthesis are shown in Table 8. In the small batch synthesis, the 4-diethylamino-2-butyn-1-ol bonded phase with the platinum catalyst showed higher surface coverage. However, in the big batch synthesis, 4-diethylamino-2-butyn-1-ol bonded phase with the free radical catalyst showed higher surface coverage.

TABLE 8 Surface Coverage for Big Batch Syntheses

Compound Name	Type of Catalyst	Carbon (%)	Surface Coverage $\alpha = \mu mol/m^2$
3-amino-3-methyl-1-butyne	Free radical	4.66	2.44
4-diethylamino-2-butyn-1-ol	Free radical	7.71	2.66
4-diethylamino-2-butyn-1-ol	Platinum catalyst	6.23	2.10

DRIFT Spectroscopy

Silica Hydride Surface Characterization

Figure 1 shows the DRIFT spectrum of the silica hydride intermediate. The sharp peak at 2250 cm^{-1} is due to the stretching vibrations of the newly formed Si—H bond, hence confirming the success of the silanization reaction. The broad peak between 3800 cm^{-1} and 3000 cm^{-1} is due to the H-bonded silanol group and adsorbed water. The band near 2890 cm^{-1} is due to the residual ethoxy group from the silanization reaction. The band near 3750 cm^{-1} is due to OH stretching vibrations from the isolated silanol groups. The rest of the peaks in the spectrum can be attributed to various fundamental vibrations of the silica matrix.

Surface Characterization of 3-Amino-3-methyl-1-butyne Bonded Phase

The DRIFT spectrum of the 3-amino-3-methyl-1-butyne bonded phase using a free radical catalyst is shown in Figure 2. The intensity of this Si—H H band is considerably reduced compared to the silica hydride intermediate, indicating that the organic group has been attached to the surface. The presence of strong C—H stretching bands in the region of 2800 cm^{-1} to 3000 cm^{-1} confirms this assumption.

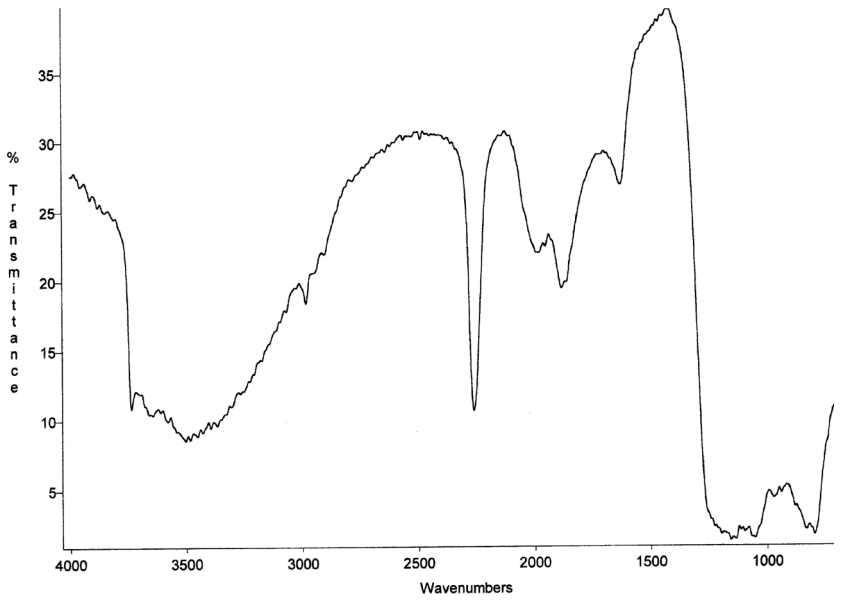


FIGURE 1 DRIFT spectrum of the silica hydride surface.

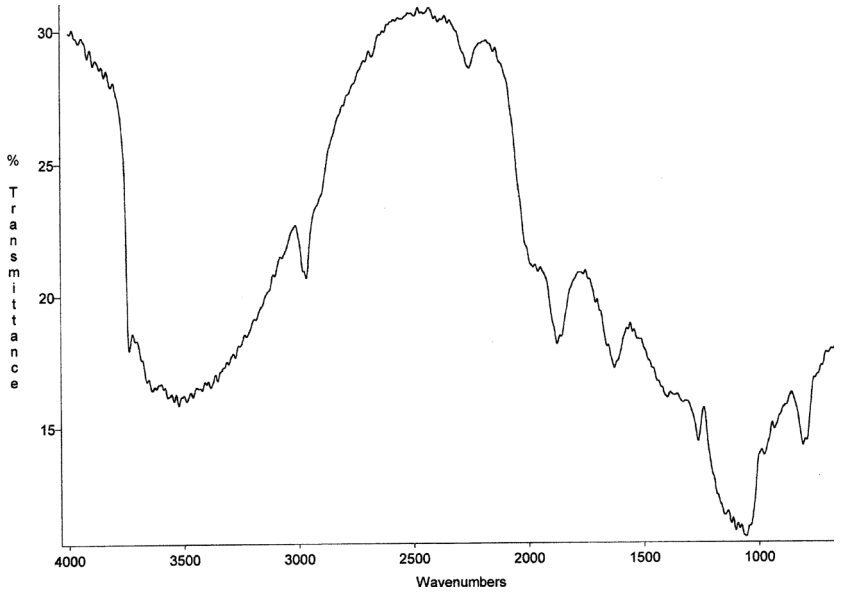


FIGURE 2 DRIFT spectrum of 3-amino-3-methyl-1-butyne bonded phase.

Surface Characterization of 3-Amino-3-methyl-1-butyne + Trypsin Bonded Phase

The DRIFT spectrum of the 3-amino-3-methyl-1-butyne + trypsin bonded phase is shown in Figure 3. A considerable change can be noticed in the DRIFT spectrum of the trypsin bonded phase compared to the 3-amino-3-methyl-1-butyne bonded phase. This is a good indication that the surface morphology has changed. Also, the sharp peak at 1700 cm^{-1} represents the C=O frequency of the amide and the carbonyl groups present on the amino acids of the protein. The other smaller peaks are due to fundamental vibrations of the various organic groups present on the protein bonded surface.

The DRIFT spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made with the platinum catalyst and 4-diethylamino-4-butyn-1-ol bonded phase made by the free radical catalyst also confirmed the bonding of an organic moiety.

Solid State ^{13}C CP-MAS NMR Spectroscopic Evaluation

Characterization of 3-Amino-3-methyl-1-butyne + Trypsin Bonded Phase

Figure 4 shows the ^{13}C CP-MAS NMR spectrum of the 3-amino-3-methyl-1-butyne + trypsin bonded phase. The peaks between 10 ppm and 60 ppm are due to the various carbons of the protein on the hydride surface. The signal at 173 ppm is due to the C=O from the amide carbonyl groups

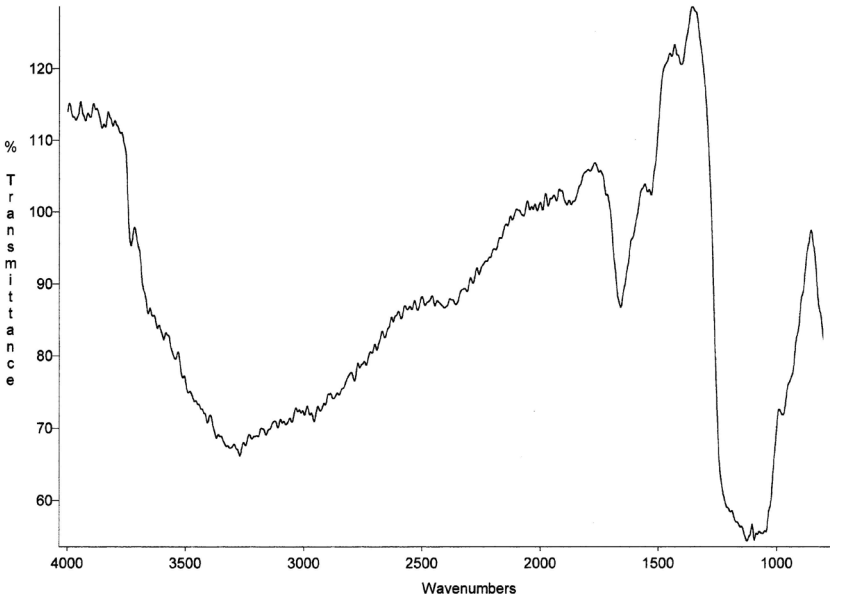


FIGURE 3 DRIFT spectrum of 3-amino-3-methyl-1-butyne + trypsin bonded phase.

present in the amino acids, hence confirming the presence of protein on the amino bonded surface.

Characterization of 4-Diethylamino-2-butyn-1-ol Bonded Phase Made by the Platinum Catalyst

Figure 5 shows the ^{13}C CP-MAS NMR spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made by the platinum catalyst. The sharp peak at 6.43 ppm is due to the methyl resonance of the bonded moiety, hence

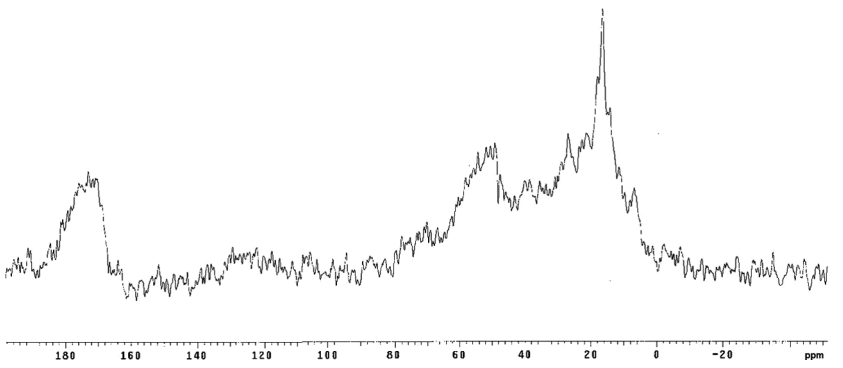


FIGURE 4 NMR spectrum of 3-amino-3-methyl-1-butyne + trypsin bonded phase.

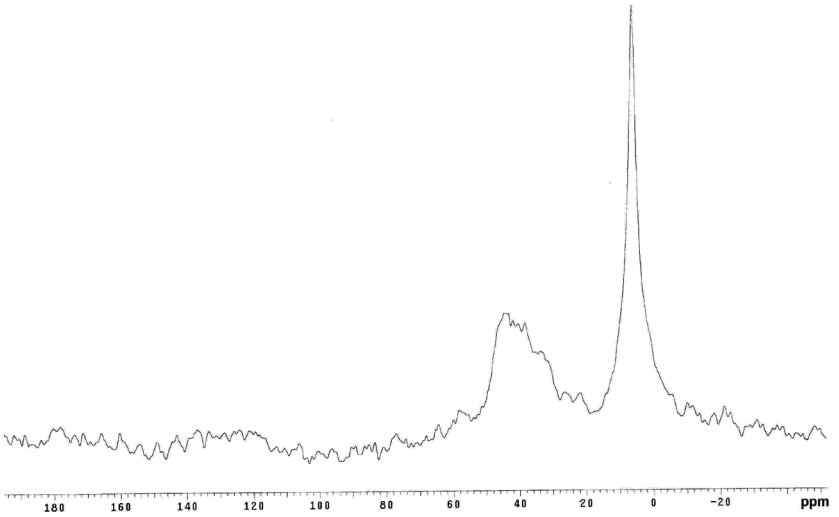


FIGURE 5 NMR spectrum of 4-diethylamino-2-butyn-1-ol bonded phase with platinum catalyst.

confirming the presence of the organic group on the silica hydride surface. The broad peak between 20 ppm and 50 ppm is due to the C—H attached to the surface of silica. Overall, the spectrum confirmed the bonding of 4-diethylamino-2-butyn-1-ol to the silica hydride surface.

The ^{13}C CP-MAS NMR spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made by the free radical catalyst was very similar to one shown in Figure 5 and confirmed bonding of the moiety to the silica surface.

Chromatographic Evaluation

Neue Test[9]

All four stationary phases were initially characterized using the Neue test in order to determine the hydrophobicity and silanophilic activity at pH 7. Two purely hydrophobic analytes, naphthalene and acenaphthene, were used to determine the hydrophobicity of the column. Amitriptyline, a polar basic compound, was used to measure the silanophilic activity of the stationary phases. To determine the dead volume of the column, uracil was used. The compounds were analyzed using 35% 20 mM K_2HPO_4/KH_2PO_4 buffer at pH 7 and 65% methanol. The hydrophobicity was measured using Equation 7, where V is the molar volume of the compound being analyzed, ν is the hydrophobicity parameter of the stationary phase and k is the retention factor of the hydrophobic compound.

$$\ln(k) = \square \times V - 3.068 \quad (7)$$

The silanophilic activity at pH 7 can be calculated using Equation 8, where S is the silanophilic activity, $k_{amitriptyline}$ is the retention factor of amitriptyline and $k_{acenaphthene}$ is the retention factor of acenaphthene. The results of the Neue test are shown in Table 9, where t_R is the retention time of the solutes in minutes.

$$S = \ln(k_{amitryptiline}) - 0.7124 \times \ln(k_{acenaphthere}) + 1.9748 \quad (8)$$

The retention time of acenaphthene and amitriptyline was less then the void volume on all four columns (determined from retention time of uracil); hence, the retention factor values were negative. The retention of the uracil might be due to the interaction of this compound with amino groups and Si−H surface which was not replaced by the organic group during the hydrosilation step. Also, the faster elution of the two hydrophobic compounds confirms the polar nature of the amino bonded phases. The longer retention time of amitriptyline could be attributed to the polar nature of the stationary phase.

Sugar Analysis

Chromatographic evaluation of all four columns was done by studying the retention of sugars on these columns. The analysis was carried out in the aqueous normal phase mode using a laser light scattering detector. Five different ratios of acetonitrile and DI water were used, starting from 95% acetonitrile and 5% water. Initially, eight sugars were analyzed on all four amino columns using only 5% water. The sugars analyzed were maltose, sucrose, glucose, fructose, lactose, xylose, raffinose, and melezitose. As sugars show typical normal phase behavior on these polar columns, the retention times of sugars were very high when 95% acetonitrile was used, making the mobile phase relatively non polar. In fact, the retention times of the disaccharides were over 60 minutes and the trisaccharides were over 90 minutes, making the analysis time very long, even for a single

TABLE 9 Results of the Neue Test

Stationary Phase	Uracil (t_R)	Acenaphthene (t_R)	Naphthalene (t_R)	Amitriptyline (t_R)
3-amino-3-methyl-1-butyne bonded phase (FR)	1.77	1.66	1.65	7.85
3-amino-3-methyl-1-butyne + trypsin bonded phase	1.74	1.70	1.72	9.75
4-diethylamino-2-butyn-1-ol bonded phase (Pt)	2.54	2.34	2.36	10.3
4-diethylamino-2-butyn-1-ol bonded phase (FR)	2.46	2.37	2.37	11.2

determination. Hence, it was decided to analyze just the monosaccharides on all four bonded phases.

For the analysis of monosccharides, the concentrations of mobile phases (acetonitrile:water) used were as follows: 95:5, 90:10, 85:15, 80:20, and 75:25. The order of elution for the sugars was based on the number of hydroxyl groups present on each sugar. In normal phase chromatography, the least polar compound is eluted first followed by more polar compounds. On all four columns, the least polar sugar, xylose, was eluted first. Glucose and fructose were eluted very close to each other. The retention of these sugars was due to the polar-polar interaction between the amino groups on the bonded phase and the OH groups of the sugars. The elution order is based on the number of hydroxyl groups on each sugar.[14] Xylose, which has only four hydroxyl groups, is eluted first, followed by fructose and glucose which have five hydroxyl groups.[15] As both fructose and glucose have five hydroxyl groups, sometimes they were eluted as a single peak. The best separation was achieved by using a lower amount of water in the mobile phase.

A study of the relationship of the retention times of the sugars with the amount of organic solvent in the mobile phase was done by plotting a retention map on all four bonded phases. As shown in Figure 6 and

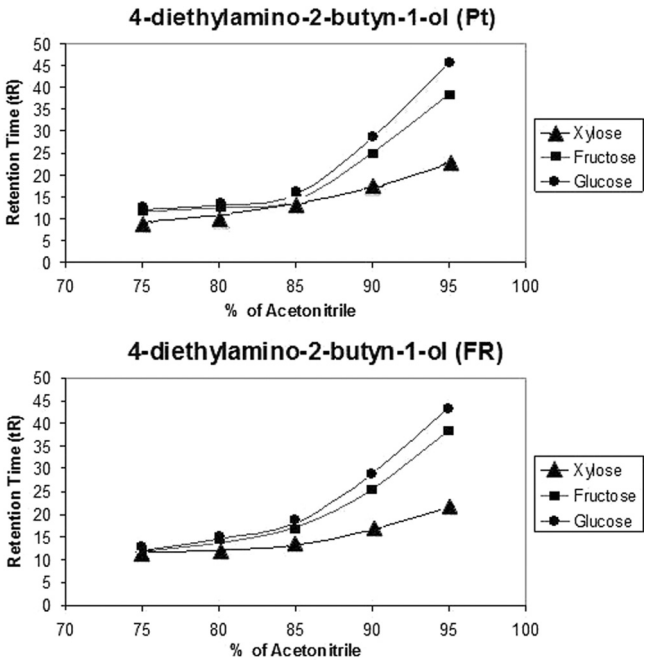


FIGURE 6 Retention map for sugar analysis on the 4-amino-2-butyn-1-ol bonded phase made with Pt (top) and the 4-amino-2-butyn-1-ol bonded phase made with free radical (bottom) using acetonitrile/water as the mobile phase.

Figure 7, the retention times of the sugars are directly proportional to the amount of organic solvent. As the amount of organic solvent increases, the retention times of the sugars increase. This type of behavior is typical of aqueous normal phase chromatography. This ANP behavior was observed on all four columns. The retention times of sugars on the 4-diethylamino-2-butyn-1-ol column using platinum catalyst and 4-amino-2-butyn-1-ol using free radical catalyst were longer, compared to the 3-amino-3-methyl-1-butyne and the protein column.

Analysis of Metformin and Antidepressants

The seven polar compounds used in the study were as follows: metformin, doxepin, amitriptyline, desipramine, nortriptyline, clomipramine, and imipramine. Uracil was used to determine the void volume. Of the seven compounds, metformin is highly polar in nature while the rest of the compounds are moderately polar. Different ratios of methanol and 20 mM K_2HPO_4/KH_2PO_4 buffer at pH 7 were used as the mobile phase. The concentrations of mobile phases (methanol/buffer) used were as follows: 25:75, 35:65, 45:55, 55:45, 65:35, 75:25, 85:15, and 95:5. Metformin

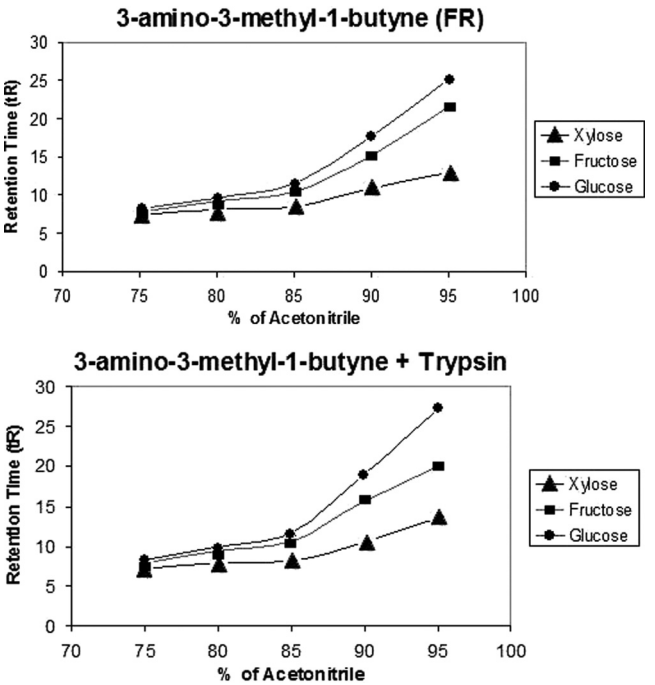


FIGURE 7 Retention map for sugar analysis on the 3-amino-3-methyl-1-butyne bonded phase made with free radical (top) and the 3-amino-3-methyl-1-butyne + trypsin bonded phase (bottom) using acetonitrile/water as the mobile phase.

showed ANP behavior on all four columns tested. Hence, retention increased as the amount of methanol increased as shown in Figures 8 and 9. This is most likely due to the interaction of the highly polar amino groups of metformin with the amino bonded phase. Also, the degree of ionization depends on the pKa of the drug and the pH of the mobile phase. The pKa of metformin is 12.4, whereas the pKa of all the other six drugs is close to 9.5. In ANP chromatography, the retention time for basic solutes increases at lower pH. The analysis was done at pH 7. Hence, the highly ionized metformin sample was retained longer on the columns as the amount of organic solvent increased in the mobile phase. It has been demonstrated that methanol, in most cases, does not provide ANP retention because of its more polar characteristics.[11]

Amitriptyline, imipramine, clomipramine, and doxepin showed reverse phase behavior on all four columns. This might be due to the hydrophobic interactions of these compounds with the stationary phase and a comparatively lower pKa value. The pKa of amitriptyline, imipramine, clomipramine

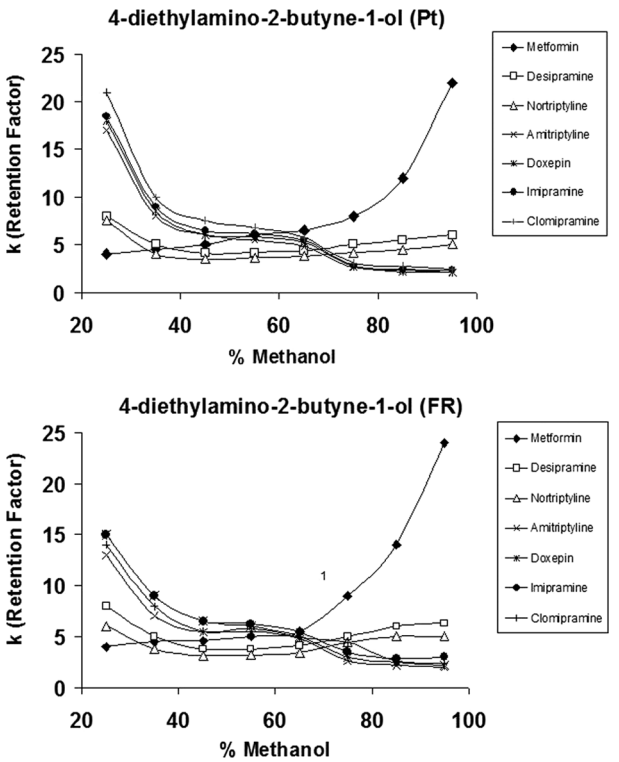


FIGURE 8 Retention map of metmorfin & antidepressants on the 4-amino-2-butyn-1-ol bonded phase made with Pt (top) and the 4-amino-2-butyn-1-ol bonded phase made with free radical (bottom) using methanol/buffer as the mobile phase.

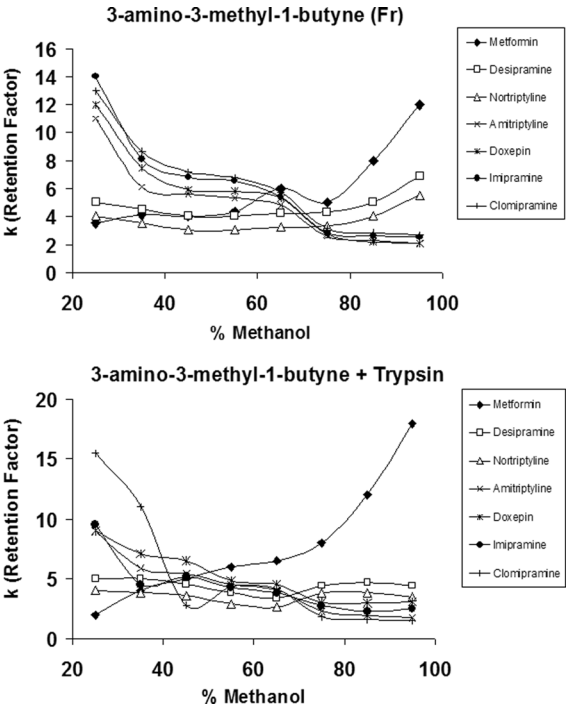


FIGURE 9 Retention map of metformin & antidepressants analysis on the 3-amino3-methyl-1-butyne bonded phase using free radical (top) and the 3-amino-3-methyl-1-butyne + trypsin bonded phase (bottom) using methanol/buffer as the mobile phase.

is 9.5 and doxepin is 9.0. Nortriptyline and desipramine, which have pKa value of 9.7 and 10.5, showed reverse phase behavior with a low amount of methanol and ANP behavior with a higher amount of methanol on all four columns. Although all four columns showed a similar retention mechanism, compounds on the protein column showed a more random pattern. This might be due to complex interactions from the various functional groups present on the amino acid chain. The retention times of pharmaceuticals on the 4-diethylamino-2-butyn-1-ol column made with the free radical catalyst was longer compared to the 4-diethylamino-2-butyn-1-ol column made with the platinum catalyst. This behavior can be attributed to the fact that the 4-diethylamino-2-butyn-1-ol column made with the free radical catalyst had higher surface coverage of the bonded phase as determined by the elemental analysis data. The retention times of pharmaceuticals on the 3-amino-3-methyl-1-butyne and the protein column were shorter compared to both the 4-diethylamino-2-butyn-1-ol columns. This was due to the difference in the column dimensions. All the pharmaceuticals under study showed good separation and peak resolution on all four columns under reversed phase conditions.

Affinity Chromatography

One of the goals of this research was to study the affinity aspect of the protein column. The column evaluated was the 3-amino-3-methyl-1-butyne bonded phase + trypsin. Type III-O trypsin inhibitor from chicken egg white was used to test the affinity aspect of the amino bonded trypsin column. The trypsin inhibitor contains 129 amino acids and was eluted through the column using two different mobile phases. A 0.01 M phosphate buffer at pH 7 was used as the attaching buffer and acetic acid was used as the eluting buffer. The phosphate buffer helps to form the affinity complex between the trypsin and the trypsin inhibitor as shown in Figure 10(a). A 20 μL sample of trypsin inhibitor was injected into the amino bonded trypsin column and run for 35 minutes using 0.01 M phosphate buffer as the mobile phase in order to form the affinity complex. The lack of a chromatographic peak under the phosphate buffer conditions is a good indication of the attachment of the inhibitor to the enzyme.

The acetic acid buffer helps to release the retained trypsin inhibitor, as shown in Figure 10(b). Once the inhibitor was attached to the protein column using the phosphate buffer, the mobile phase was changed to

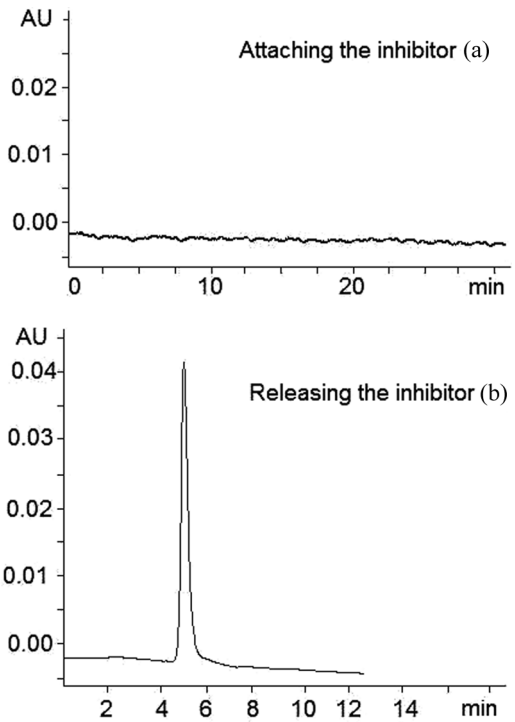


FIGURE 10 Affinity chromatography of trypsin inhibitor on trypsin column. Mobile phase for attaching the inhibitor is phosphate buffer at pH 7 (a) and acetic acid buffer is used for releasing the inhibitor (b).

the acetic acid buffer. A sharp peak for the trypsin inhibitor was observed after 5.64 minutes, indicating the release of the inhibitor from the coupled trypsin enzyme. Due to the highly specific nature of affinity chromatography, it can be confirmed that the trypsin enzyme was attached to the 3-amino-3-methyl-1-butyne bonded phase.

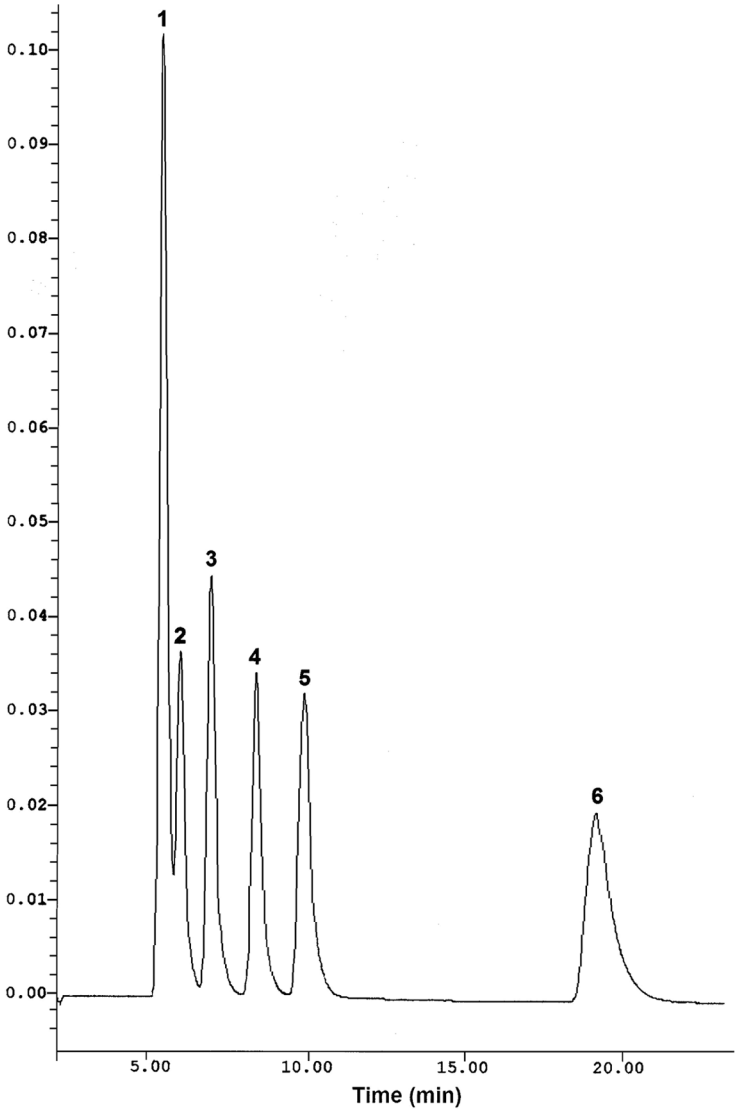


FIGURE 11 Separation of polar compounds on the 4-diethylamino-2-butyn-1-ol bonded phase (Pt). Mobile phase: 45% (20 mM K_2HPO_4/KH_2PO_4 buffer pH 7): 55% (Methanol), UV: @ 254 nm. Samples: 1. Nortriptyline; 2. Desipramine; 3. Clomipramine; 4. Doxepin; 5. Imipramine; 6. Metformin.

Overall Column Performance

Figure 11 shows a typical separation of mixture of polar compounds on the 4-diethylamino-2-butyn-1-ol bonded phase made with the platinum catalyst. Each sample was injected three times before running the mixture; the difference in the retention times was +/−0.01 min. Hence, the results indicated good reproducibility. All four columns showed good separation and peak resolution (results not shown). On all four stationary phases, the best separation of the polar compounds was obtained when the mobile phase had almost equal amounts of buffer and organic solvent.

CONCLUSION

The two main goals of this research were the synthesis and the characterization of the stationary phases. The spectroscopic characterization of the amino bonded phases and the protein column using DRIFT and solid state NMR confirmed the attachment of the desired organic group. The elemental analysis indicated that the hydrosilation reaction using the free radical catalyst provided better surface coverage of the bonded phase. The sugars showed ANP behavior on all four columns and, hence, confirmed the polar nature of the bonded phase as determined by the Neue test. The sugars were retained much longer on the 3-amino-3-methyl-1-butyne bonded phase and the protein bonded phase. This behavior was attributed to the presence of the free amino groups in both the bonded phases. Also, of the two 4-diethlamino-2-butyn-1-ol columns, the one synthesized using a free radical catalyst was able to retain sugars longer. This is due to the better surface coverage of the bonded phase as evidenced by the elemental analysis results.

When metformin and tricylic drugs were analyzed, metformin showed ANP behavior on all four columns. Amitriptyline, doxepin, imipramine, and clomipramine showed classic reverse behavior where the retention of the solute decreases as the amount of organic solvent increases. Nortriptyline and desipramine showed both reverse phase and ANP behavior under different mobile phase conditions. The affinity aspect of the trypsin column was confirmed by attaching and eluting trypsin inhibitor under different mobile conditions.

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