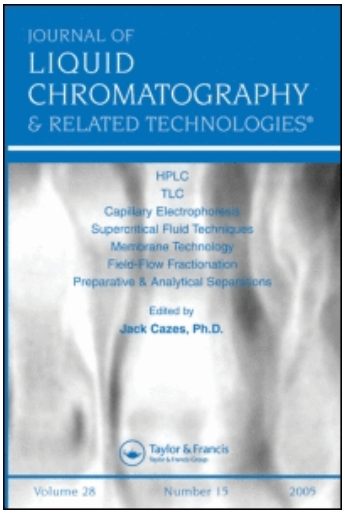

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REVERSED PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BASIC DRUGS ON A SILANOL DEACTIVATED SUPPORT

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ABSTRACT

When polar and non-polar basic drugs were separated by reversed phase HPLC, a short-chain silanol deactivated (SCD-100) reversed phase column gave superior results over a standard C18 reversed phase column. The nature of silane, the type of silica and the chemistry of endcapping influenced chromatographic behavior. The peaks in the chromatogram obtained from this column had excellent peak shapes and eluted at predictable retention times, indicating that no silanols remained on the surface. The mobile phase was composed of phosphate buffer at pH 3.5, with variable amounts of methanol. All applications were isocratic without the addition of silanol suppressing reagents.

INTRODUCTION

Small basic molecules are often difficult to analyze by reversed phase chromatography. They adsorb to the stationary

* This work was presented at the 39th Pittsburgh Conference, New Orleans, February 1988.
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phase, resulting in late-eluting undefined peaks which are frequently non-symmetrical and tailing. At worst, the solutes are permanently retained on the stationary phase, even at high concentrations of organic solvent. Although this tailing effect is evident on both organic polymeric (1) and silica-based supports (2), on silica-based reversed phase columns, the tailing effect has been attributed to the interaction between uncapped silanols and basic analytes. This interaction mechanism is believed to be occurring via ion exchange and/or hydrogen bonding (3) of the solutes with some unreacted silanols which generally remain on the surface, even with good derivatization techniques.

Silanol suppressing reagents like triethylamine have been added to the mobile phase to alleviate the interaction problems. These reagents cause basic solutes to elute with shorter retention times and improved peak symmetry. Unfortunately, the addition of silanol suppressing reagents has several disadvantages. First, retention times of the solutes are dependent on the concentration of the silanol suppressing reagent. Usually a threshold concentration of suppressing reagent is needed for the bases to elute at the hydrophobically correct retention times (without silanol interactions). This threshold value varies between different types of silicas (4), different manufacturers' synthetic techniques and different batches. The result is irreproducibility in retention times and capacity factors, which is a particular problem in quality control methodology.

Secondly, long equilibration times are needed before analysis and silanol suppressing reagents are difficult to remove after analysis. When triethylamine is used, for example, complete restoration of the reversed phase column is not observed until 1 hr of washing with aqueous solvent. Longer alkylamines take even

more time to remove from columns (3). Therefore, loss of time is expected if subsequent analyses require different solvent systems.

Thirdly, in preparative HPLC it is frequently difficult to isolate the solute from the silanol suppressing reagent upon collection. If the basic drug, for example, has to be obtained in a pure form free of silanol suppressing reagents, another separation step must be introduced.

This paper describes a reversed phase support that has been optimized by the choice of silane, silica and endcapping in such a way that basic drugs can be analyzed in the absence of both silanol suppressing reagents and high percentages of organics. The basic analytes elute as symmetrical peaks with high plate counts.

EXPERIMENTAL

Chemicals

Methanol was obtained from American Burdick & Jackson (Muskegon, MI) and potassium phosphate monobasic from Mallinckrodt (Paris, KY). Distilled grade water was used. The inorganic salt and water mixture was filtered with a 0.45 μm Nylon-66 filter from Rainin Instrument Co, Inc. (Woburn, MA). Triethylamine (gold label), procainamide hydrochloride, N-acetylprocainamide hydrochloride and salicylic acid (sodium salt) came from Aldrich Chemical Co, Inc. (Milwaukee, WI). Caffeine, phenylthiohydantoin-arginine hydrochloride, phenylthiohydantoin-glycine hydrochloride, lidocaine, amitriptyline hydrochloride, niacinamide, pyridoxine monohydrochloride, riboflavin, thiamine hydrochloride, cyanocobalamin, cytosine, 5-methylcytosine, uracil and thymine

were obtained from Sigma Chemical Co. (St. Louis, MO). The mobile phase was adjusted to pH 3.5 with hydrochloric acid after addition of methanol or triethylamine.

Apparatus

All reversed phase supports were synthesized by SynChrom, Inc. (Lafayette, IN). The short-chain silanol-deactivated reversed phase column has been denoted SynChropak SCD-100. The C18 column was the SynChropak RPP-100. All silica supports were 100Å pore size and 5 μm particle size and columns were 250 x 4.6mm ID. A model V4 variable wavelength absorbance detector from ISCO (Lincoln, NE) was operated at 254 nm. The Constametric IIIg HPLC pump was obtained from LDC/Milton Roy (Riviera Beach, FL). The injector was a Rheodyne (Anspec, Ann Arbor, MI), model 7125, equipped with a 20 or 50 μl loop. The components had been interfaced to an Apple Computer, model IIe, and all applications were run isocratically. The recorder was a Linear Model 1200 (Serco Inc., Deerfield, IL). Full absorbance scale on the recorder was usually 0.1.

RESULTS

Silane Selection

The type of silane that would give maximum covalent bonding with the silanols was investigated by comparing experimental silanes with the popular octadecylsilane. A SynChropak RPP-100 column, which contains 100Å silica bonded with C18 without endcapping, was used as the reference. In each case, the silanes were attached with optimun bonding technology. To test the performance of the alkyl bonded stationary phases, retention

times, peak heights and peak symmetries of a selected group of solutes were evaluated.

The test probes included basic, acidic and neutral drugs and consisted of procainamide, N-acetylprocainamide, salicylic acid and caffeine. At a mobile phase pH of 3.5, the two basic drugs, procainamide and N-acetylprocainamide (NAPA) are positively charged. NAPA has been a particularly troublesome solute on commercial reversed phase columns (3). Salicylic acid has a low pKa and is negatively charged at pH 3.5. This compound represents a class of acidic components which has often caused problems on endcapped C18 columns (3). Caffeine, the neutral compound, was the internal standard because it is considered to be generally well-behaved on reversed phase columns. Caffeine was also used to monitor column packing performance. The quantity of the different drugs injected onto the support ranged from 0.5-1 µg. An identical amount of each particular drug was injected onto each column; therefore, peak height could be indicative of efficiency.

In Figure 1, the elution of the test probes on the C18 column and on a short-chain reversed phase column are compared. The two basic drugs, procainamide and NAPA, look poor on the C18 column. Both peaks are tailing substantially, the peak heights are short, and the retention times are longer than expected. NAPA, in fact, elutes after salicylic acid. Apparently, the basic probes are interacting with the stationary phase in an uncontrolled fashion instead of via a purely hydrophobic partitioning mechanism.

The stationary phase bonded with a short-chain silane functioned much better, as is evident in Figure 1b. NAPA is now eluting before salicylic acid. Both procainamide and NAPA are reasonably tall and sharp although they are still tailing slightly. Even when using generally successful bonding

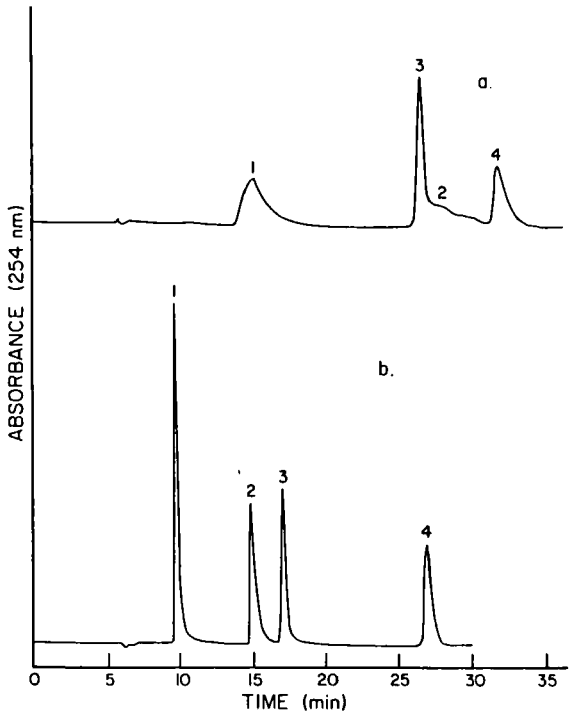

Figure 1. Influence of silane on peak shape. Columns: a. SynChropak RPP 100(C18); b. short-chain RP, 100Å, 250 x 4.6 mm I.D. Mobile phase: 30% methanol, 0.04M potassium phosphate, pH 3.5. Flowrate: 0.5ml/min. Sample: 1. Procainamide, 2. NAPA, 3. Salicylic acid, 4. Caffeine.

techniques, it is obvious that the type of silane determines the efficacy of the reversed phase support in the separation of basic solutes. It would appear that the shorter chain silane covers the silica surface better, leaving fewer free silanols on the stationary phase.

Silica Selection

To obtain a reversed phase support that does not adversely interact with basic molecules, not only is the choice of silane

crucial, but also the type of silica. Such importance of the silica has been addressed previously (5). Quite simply, some silica supports bond with silanes in a higher yield than other silicas. One explanation for this effect is that the silanols are more evenly distributed over the surfaces of certain silicas, and thus the silanes can also cover more of the surface, leaving fewer silanols exposed. It has also been suggested that it is not the residual silanols that cause the adsorption of analytes, but rather the uncovered siloxane groups on the silica. A larger number of evenly distributed silanols results in a higher silane bonding yield with better coverage of the siloxanes. Another explanation is that certain silicas contain silanols which are more acidic than the average silanol (5) and these silanols react poorly with silanes. Based on observations of silane bonding, silicas have been classified into two types, sometimes denoted A and B types (6). The A type is believed to contain more isolated acidic silanols which are not evenly distributed.

With this in mind, one silica from the A type and one from the B type were compared by bonding with the short-chain silane. The short-chain silane was attached to each silica with identical bonding chemistry. The two types of silica had 100Å pore diameters with 5 μm particle size and 200 m^2/g surface area.

Figure 2 shows that the silica type B behavior was superior to that of silica type A for this application. On the latter column, the procainamide and NAPA peaks are broad, short and severely tailing, with NAPA eluting after salicylic acid.

A reversed phase column made from such a type A silica could only be used for the analysis of basic solutes with the addition of a silanol suppressing reagent to the mobile phase. Figure 3 shows the effect of adding 0.008 M triethylamine (TEA) to the

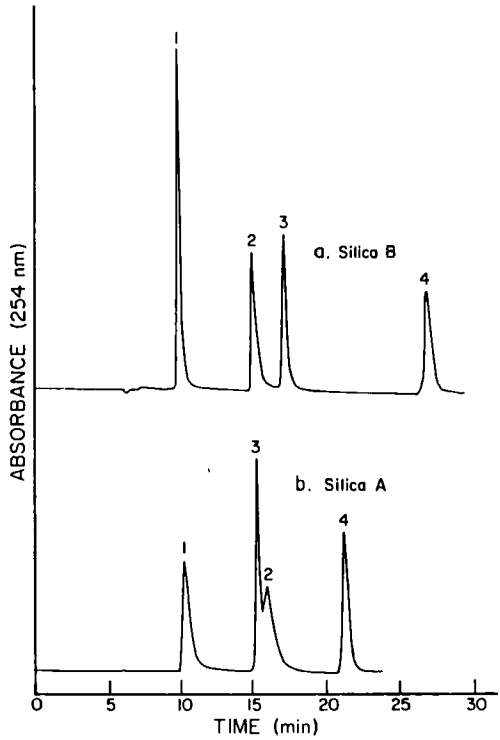

Figure 2. Influence of silica type on peak shape. Columns: short-chain RP, 100Å a. silica type B; b. silica type A. Conditions as in Fig. 1.

mobile phase on this column. Under these conditions, NAPA is eluting before salicylic acid and the peak shapes have improved considerably, peak tailing has diminished, and the peaks are taller and narrower. Some tailing is still present, however, indicating that the support is still not optimum for the basic drugs. Such earlier elution of basic probes after addition of TEA is an indication that the silanols are being suppressed.

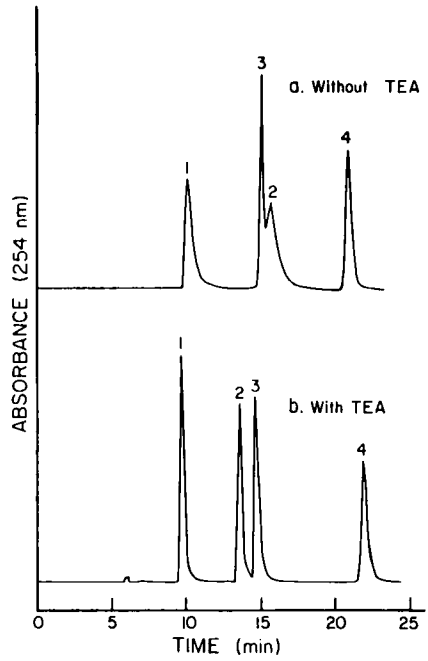

Figure 3. Influence of silanol suppressing agent on an inadequately bonded short-chain reversed phase support. Column: short-chain RP, 100Å, Silica type A. Conditions as in Fig. 1 except 0.008M triethylamine (TEA) is added to the mobile phase in b.

Endcapping

Although the performance of the support seen in Figure 2a was similar to that obtained using silanol suppressing reagents on other supports (Fig. 3b), procainamide and NAPA were still tailing slightly. This suggested the necessity of an additional synthetic step.

The remaining silanols on the support seen in Figure 2a were deactivated with an endcapping reagent. Figure 4 shows a chromatogram resulting from the injection of the four drug probes. When comparing these figures, the two basic drugs, procainamide and N-acetylprocainamide elute as sharp, tall, symmetrical peaks

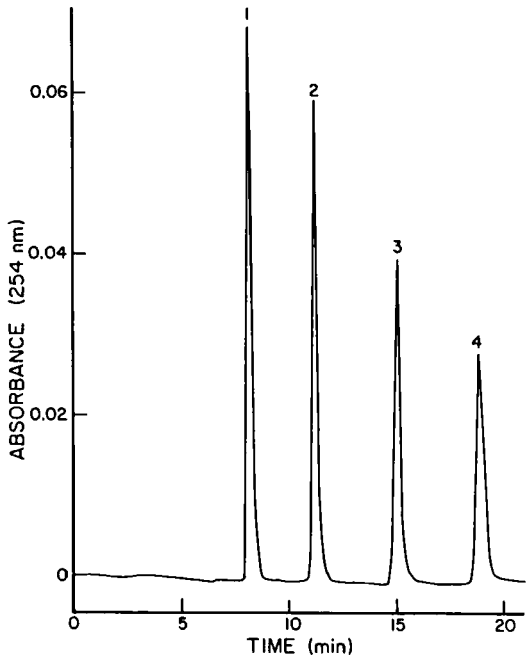

Figure 4. Basic drugs on a short-chain silanol deactivated support. Column: SynChropak SCD-100, 250 x 4.6 mm I.D. Conditions as in Fig. 1.

with shorter retention times than on the support before deactivation. The acidic salicylic acid also elutes as a sharp peak; however, its retention has not been substantially affected by the silanol deactivation.

When positively charged triethylamine was added to the mobile phase (data not shown), the acidic salicylic acid decreased in retention time, while the three nitrogen-containing drugs remained the same on this column. The shift in salicylic acid retention may stem from formation of an ion pair between the salicylic acid and the positively charged TEA. Significantly, the lack of change in retention for the basic drugs indicates a well-deactivated support upon which a silanol suppressor does not improve.

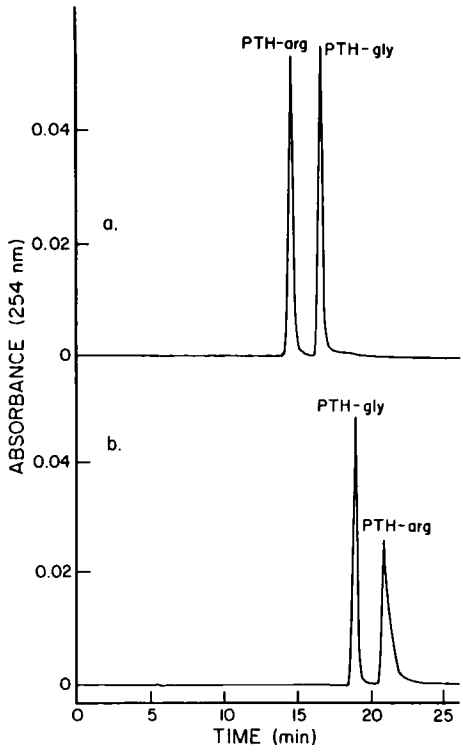

Figure 5. Comparison of analyses of PTH amino acids on standard C18 and short-chain silanol deactivated reversed phase columns. Columns: a. SynChropak SCD-100; b. SynChropak RPP-100 (C18). Conditions as in Fig. 1.

Analysis of PTH Amino Acids

Figure 5 illustrates the separation of two PTH-amino acids on the silanol deactivated column (a) and on the reference 100Å C18 column (b). The same amounts of the PTH-amino acids were injected on both columns. Because partition parameters (7) and free energy transfer parameters (8) suggest that arginine is more hydrophilic than glycine at pH 3.5, PTH-arg should elute before PTH-gly if the two PTH-amino acids are eluting strictly according to hydrophobic partitioning. This is the case on the silanol

deactivated support. In contrast, the PTH-arg elutes as a tailing and a fairly short peak after PTH-gly on the C18 column. The PTH-arg is apparently interacting with residual silanols on this reference column. Therefore, hydrophobicity measurements, such as the log p(octanol) partition coefficients of drugs (9), may be better correlated to chromatographic retention data on the silanol deactivated column than on one which is not deactivated.

Amitriptyline Analysis

Figure 6 illustrates the chromatographic characteristics of lidocaine and amitriptyline, two other hydrophobic basic drugs, on the short-chain silanol deactivated column. Amitriptyline is very hydrophobic and normally requires a large amount of organic solvent to elute from a C18 column (10). In this example, a 60% methanol solution enabled the tricyclic amitriptyline to elute in 7 min. Both peaks are tall, narrow and symmetrical despite the basic nature of the drugs. With less methanol, these hydrophobic drugs would still have eluted in a reasonable time. The short-chain reversed phase column is advantageous because less organic solvent is needed for elution, resulting in a savings in cost of the organic solvent as well as time.

B Vitamins

B vitamins have frequently been difficult to analyze and separate by reversed phase chromatography, often requiring ion pairing reagents or silanol suppressors to be added to the mobile phase (11). Although the B vitamins all contain amine groups, they range from charged and hydrophilic to uncharged and hydrophobic. Thiamine and pyridoxine are charged at pH 3.5, whereas vitamin B12 is hydrophobic and should elute last. In the

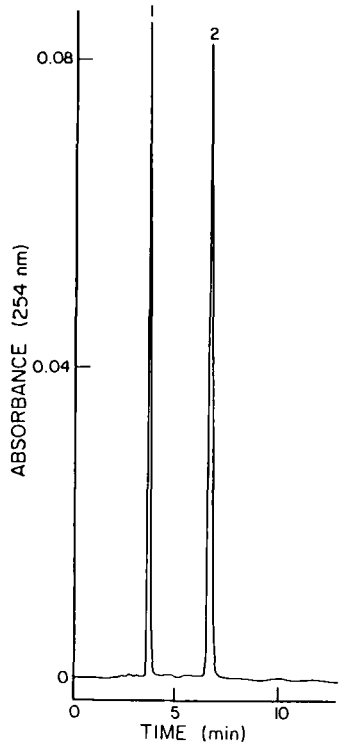

Figure 6. Analysis of highly hydrophobic basic drugs. Column: SynChropak SCD-100, 250 x 4.6 mm I.D. Mobile Phase: 60% methanol, 0.04 M potassium phosphate, pH 3.5. Flowrate: 1 ml/min. Sample: 1. lidocaine, 2. amitriptyline.

separation of B vitamins depicted in Figure 7, it can be seen that all of the vitamins are resolved in this isocratic methanolic aqueous mobile phase at pH 3.5. Not only is the order of elution by hydrophobicity, but the peaks are well-shaped and symmetrical.

Pyrimidines

The pyrimidines, the structural units of DNA and RNA, are another set of weak bases. At pH 3.5, cytosine and

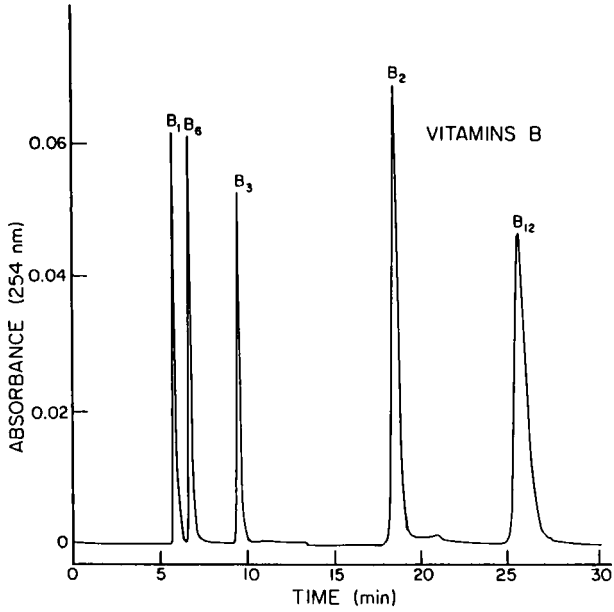

Figure 7. Analysis of B vitamins. Conditions as in Fig. 4.

5-methylcytosine are positively charged and uracil and thymine are neutral. 5-Methylcytosine and thymine each contain one methyl group more than their respective counterparts, cytosine and uracil. On the short-chain deactivated column, as illustrated in Figure 8, the pyrimidines elute as symmetrical peaks in the predicted order of hydrophobicity. On the C18 column (data not shown), both cytosine and 5-methylcytosine tailed somewhat and 5-methylcytosine eluted after uracil.

CONCLUSIONS

The quality of separation of basic drugs on a short-chain silanol deactivated reversed phase column was greatly improved

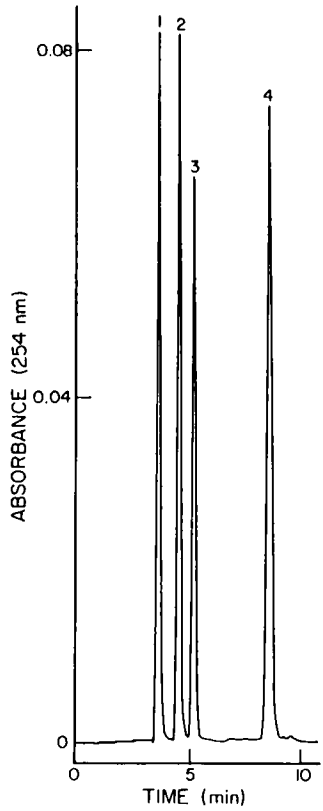

Figure 8. Analysis of pyrimidines. Mobile phase: 0.04 M potassium phosphate, pH 3.5. Flowrate: 1 ml/min. Sample: 1. cytosine, 2. 5-methylcytosine, 3. uracil, 4. thymine.

over that on a standard C18 reversed phase column. Silane, silica type and endcapping each had a role in eliminating nonspecific interactions of basic solutes with the support. Simple mobile phases, without silanol suppressing reagents, could be used, as evidenced by the excellent separation of procainamide and N-acetylprocainamide which was obtained isocratically with phosphate buffer at pH 3.5 containing low percentages of methanol. PTH-arginine, which is a very sensitive probe for detecting

uncapped silanols, definitively confirmed that few, if any, silanols remained on this deactivated support. Consequently, the use of silanol suppressors in the mobile phase was not necessary.

All of the therapeutic drugs analyzed, including the antiarrhythmic agents, procainamide, N-acetylprocainamide and lidocaine, and the antidepressant, amitriptyline, eluted as sharp bands without tailing on the silanol deactivated column. More weakly basic compounds, such as the nutritionally important B vitamins and the pyrimidines, also eluted as well-resolved, symmetrical peaks.

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