Synthesis and characterization of novel polar-embedded silica stationary phases for use in reversed-phase high-performance liquid chromatography

Haibo Wang a, Lei Chen b,1, Xiuling Tang a,1, Yanyan Jia b, Guangqing Li c, Xiaoli Sun b,*, Aidong Wen b,*

a Department of Chemistry, School of Pharmacy, Fourth Military Medical University, Xi’an 710032, China
b Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi’an 710032, China
c Dima Outai Technology Development Center, Beijing 100029, China

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A B S T R A C T
We have developed a series of new C10 dipeptide stationary phases via a simple and effective synthetic method. The preparation of the new phases involves the synthesis of silanes and the surface modification of silica. Chromatographic evaluations of these columns were performed using the Engelhardt, Tanaka, and Neue test mixtures. The applicability of these new stationary phases was also evaluated using a series of diagnostic probes including acids, bases or neutral compounds and several generic applications. These new C10 dipeptide stationary phases showed excellent hydrolytic stability over a wide pH range. Like other existing amide-embedded columns, these new stationary phases exhibit higher retention for polar and hydrophilic compounds and different selectivity as compared to conventional C8 columns. These new phases are compatible with 100% aqueous mobile phases, and also provide high column efficiency and good peak shapes for both acidic and basic compounds.

1. Introduction

Reversed-phase liquid chromatography (RPLC) is the most popular and efficient separation technique due to its high separation efficiency, excellent reproducibility and MS compatibility [1–3]. A very large number of reversed-phase liquid chromatography stationary phases are commercially available on the market and new ones are being introduced regularly. However, current silica-based RPLC packings have several drawbacks such as peak tailing of basic compounds [4,5] and dewetting in highly aqueous environments [6–10], which impede their use in certain applications.

During the past few decades, much work has focused on improving the chemical stability of silica-based stationary phases [11]. Several new stationary phases containing endcapped or embedded polar groups have been developed, providing users with alternative selectivities for more difficult chromatographic separations involving polar, highly basic and ionizable compounds in reversed phase conditions [5,12]. Stationary phases with embedded amide groups were first developed using a two step modification process where aminopropyl silica was acetylated to form the polar amide group [13–16]. The procedure involving a two step modification process suffers from the difficulty of achieving a high yield of acylation groups as it is well known that the conversion of amine groups to amide in the second modification step is not quantitative. As a result, a heterogeneous surface is obtained having both acetylated and underivatized amine groups. The presence of some residual aminopropyl groups causes undesirable interactions during the chromatographic separations.

As an alternative approach, O’Gara et al. [17] reported the preparation of a new C8 phase containing embedded carbamate groups using a single-step modification process. This approach was based on the prior synthesis of the appropriate monofunctional chlorosilane containing polar functional carbamate groups, followed by chemical modification of the silica surface with this chlorosilane reagent and subsequent endcapping. The methodology of preparing an organosilane with the desired substituents and then bonding it to the silica surface is advantageous over the two step modification process because the absence of a second reaction step allows the obtaining of a homogeneous composition attached to the silica surface.

To the best of our knowledge, stationary phases containing dipeptide functionalities have not been reported. Here, we have prepared a series of new stationary phases containing embedded dipeptide groups by a single step modification process (Fig. 1), based on synthesis of trifunctional or monofunctional dipeptide alkoxy silanes by a proprietary chemical process, followed by modification of the bare silica and further endcapping. We attempted to characterize these phases using a series of assays designed to elucidate fundamental chromatographic behavior and have included
2.2. Determination of metal impurities in silica gels

Water (5 g) was added to 1 g of silica gel in a PTFE beaker. A 5 mL volume of a 50% aqueous solution of hydrofluoric acid was added to the silica gel to dissolve it. The solution was heated on a hot plate and evaporated to dryness. The residue was dissolved using 0.25 mL of concentrated HNO₃, and the solution diluted to 25 mL with water. The final solution was analyzed using an inductively coupled plasma mass spectrometer (PMS 200, Yokogawa Analytical System, Tokyo, Japan). The total metal content is less than 30 ppm.

2.3. Synthesis of organosilanes and stationary phases

2.3.1. Preparation of 2-undecanamidoacetic acid

Undecanoyl chloride (1.1 equiv) and Et₂N (2.0 equiv) were added to dry CH₂Cl₂ (50 mL) with vigorous stirring in an ice bath. To this mixture, a solution of glycine (1.0 equiv) in dry CH₂Cl₂ (20 mL) was added dropwise over a period of 20 min at 0 °C. The mixture was warmed to room temperature and stirred for 2 h. Then, the reaction mixture was treated with water and extracted with CH₂Cl₂. The organic layer was dried over anhydrous Na₂SO₄ and concentrated. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (3:1) as eluant, giving a colorless oil product. ¹H NMR (500 MHz, CDCl₃) δ 0.84 (t, 3H), 1.29 (m, 14H), 1.58 (m, 2H), 2.15 (m, 2H), 4.16 (s, 2H). Calc. C% 64.16, H% 10.36, N% 5.76; found C% 64.10, H% 10.38, N% 5.71.

2.3.2. Preparation of N-(2-oxo-2-(3-(dimethyloxymethyl)propylamino)ethyl)undecanamide

A three-neck round-bottomed flask equipped with a mechanical stirrer, a refluxing condenser and a dropping funnel, was charged with 2-undecanamidoacetic acid (10.0 mmol), N,N′-dicyclohexylcarbodiimide (DCC) (2.1 g, 10.0 mmol), 4-dimethylamino pyridine (DMAP) (70 mg, 0.57 mmol), and dichloromethane (100 mL). 3-Aminopropyltrimethoxysilane (10.0 mmol) was then added dropwise to the flask. The mixture was stirred at room temperature for 3 h. The reaction solution was filtered, the filtrate was washed with 3 × 20 mL 20% sodium carbonate solution, and the organic layer was dried by anhydrous magnesium sulfate. The solvent was removed under reduced pressure to afford a crude product, which was further purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate, 3:7) to yield a white solid (3.54 g, 83.3%), m.p. 58–59 °C. ¹H NMR (500 MHz, CDCl₃) δ 0.57 (m, 2H), 0.86 (t, 3H), 1.29 (m, 14H), 1.60 (m, 2H), 2.09 (t, 2H), 3.21 (q, 2H), 3.60 (s, 9H), 4.12 (b, 2H). Calc. C% 56.40, H% 9.96, N% 6.92; found C% 56.33, H% 9.87, N% 6.98. N-(2-oxo-2-(3-(dimethyloxymethyl)propylamino)ethyl)undecanamide and N-(2-oxo-2-(3-(dipropylmethoxymethyl)propylamino)ethyl)undecanamide can be prepared by a similar method. N-(2-oxo-2-(3-(dimethyloxymethyl)propylamino)ethyl)undecanamide: m.p. 53–55 °C. ¹H NMR (500 MHz, CDCl₃) δ 0.13 (m, 2H), 0.85 (t, 3H), 1.29 (m, 14H), 1.31 (m, 2H), 1.58 (m, 2H), 1.62 (m, 2H), 2.15 (t, 2H), 3.20 (q, 2H), 3.55 (s, 3H), 4.07 (b, 2H). Calc. C% 61.24, H% 10.82, N% 7.52; found C% 61.27, H% 10.76, N% 7.55. N-(2-oxo-2-(3-(dipropylmethoxymethyl)propylamino)ethyl)undecanamide: m.p. 67–69 °C. ¹H NMR (500 MHz, CDCl₃) δ 0.95 (m, 12H), 0.86 (t, 3H), 1.26 (m, 14H), 1.3 (m, 2H), 1.57 (m, 2H), 1.60 (m, 2H), 1.82 (m, 2H), 2.19 (t, 2H), 3.22 (q, 2H), 3.60 (s, 3H), 4.09 (b, 2H). Calc. C% 64.43, H% 11.28, N% 6.53; found C% 64.48, H% 11.25, N% 6.55.

2. Experimental

2.1. Reagents and materials

Organic silane reagents were obtained from Gelest (Tullytown, PA, USA) or Silar Laboratories (Wilmington, NC, USA). Hydrofluoric acid and nitric acid were of trace analysis grade from Kanto Chemicals (Tokyo, Japan). The acetonitrile and methanol used in these analyses were HPLC grade from VWR Scientific Products (San Dimas, CA, USA), and the de-ionized water was prepared using an E-Pure water purification system (Barnstead/Thermolyne, Dubuque, IA, USA). All reagents were of the highest possible purity and were purchased from Sigma–Aldrich Inc. (Milwaukee, WI, USA). The spherical silica particles, with a mean particle size of 5 μm, mean pore diameter of 10 nm, and BET surface area of 400 m² g⁻¹, were supplied from AGC Si-Tech Co. Ltd. (Tokyo, Japan).

several applications that may further illustrate the differences between these phases and conventional C₁₈ phases. This evaluation will provide insight into the usefulness and limitations of polar-modified columns for the LC chromatographer.

Fig. 1. Preparation of monofunctional C₁₀ dimethyl and disopropyl dipeptide phases.
2.3.3. Preparation of C_{10} dipeptide bonded phases

Three different stationary phases were prepared using the homologous series of the C_{10} dipeptide silanes (trimethoxy, dimethylmethoxy, and diisopropylmethoxy). The derivatized silicas were named polymeric C_{10} dipeptide, C_{10} dimethyl dipeptide, and C_{10} diisopropyl dipeptide, respectively. A suspension of 10 g of silica gels in 50 mL of concentrated HCl was heated at 100 °C for 16 h, and then cooled to 25 °C. The silica gels were collected, washed with water until free from acid, and dried under vacuum at 140 °C for 8 h. An excess of C_{10} dipeptide silane (13.5 μmol m^{-2} of bare silica), dry pyridine (a calculated equivalent of 12 μmol of reagent per square meter of silica surface) and n-decane (50 mL) were added. The suspension was mechanically stirred and refluxed under a nitrogen atmosphere for 24 h. The modified silica was filtered and washed with toluene, dichloromethane, THF, methanol, and a water–methanol mixture. The bonded silica gels were then hydrolyzed with 0.1% trifluoroacetic acid in 5:1 MeOH:water at room temperature for 16 h. The material was filtered and washed with acetone, methanol, and a water–methanol mixture. The bonded silicas were dried under vacuum at 80 °C for 8 h prior to an endcapping reaction. The modified silica was endcapped using a conventional liquid phase reaction. Briefly, the reaction was performed by refluxing 10 g of each modified silica with a stoichiometric excess of endcapping reagent i.e., 8 mL of hexamethyldisilazane (HMDS) in 50 mL of dry n-decane with 2 mL of pyridine. After the mixture was refluxed for 16 h, the silica gels were filtered and washed repeatedly with toluene, dichloromethane, THF, methanol, a water–methanol mixture, water, and finally with methanol. All materials were dried under vacuum at 80 °C for 8 h prior to characterization or packing.

2.4. Chromatographic measurements

The resulting bonded phase was packed into two individual stainless steel tubes (150 mm × 4.6 mm I.D.) by conventional high pressure slurry packing procedures. The columns were used for evaluation of the chromatographic performance. All experiments were carried out at 298 K. All solvents were filtered and degassed before use. The HPLC system used was a model Agilent 1200 series (Palo Alto, CA, USA) consisting of an Agilent 1200 in-line degasser, an Agilent 1200 autosampler, an Agilent 1200 column thermostat set to 30 °C, an Agilent 1200 quaternary pump, and an Agilent 1200 variable wavelength detector. Agilent Chemstation software was used for data acquisition and analysis. Uracil was used as a void volume marker.

3. Results and discussion

3.1. Preparation of the monofunctional and trifunctional C_{10} dipeptide phases

The preparation of the new monofunctional C_{10} dipeptide phases is outlined in Fig. 1. In the first step of the chemical modification, the methoxy group from the silane reacts with the surface silanols, yielding the modified phases (A). Pyridine was used as a basic catalyst to enhance the surface coverage of the silica surface. The modified silicas were purified with repeated washings to remove physically adsorbed silane. In a further step, the modified surfaces were endcapped through reaction with a stoichiometric excess of hexamethyldisilazane (HMDS). The residual silanols were blocked by substitution by a –SiMe_{3} moiety, as represented in (B).

The concentration of the organic groups attached to the silica surface was calculated from the carbon percentages and the BET surface area of the bare silica. At least three batches were made for each material. Microanalyses are in good agreement with the proposed formulation. The modification process yielded modified silicas with a mean ligand surface concentration of 3.63, 3.75, and 2.07 μmol m^{-2} for the polymeric C_{10} dipeptide phase, C_{10} dimethyl dipeptide phase, and C_{10} diisopropyl dipeptide phase, respectively (Table 1). A predictable lower surface coverage was obtained for the C_{10} diisopropyl dipeptide phase due to the presence of the diisopropyl groups. This μ value is similar to the reported value of 2.1 μmol m^{-2} for the C_{14} diisopropyl amide stationary phases [16]. This lower surface coverage is typical in the preparation of phases using this type of sterically protected silane [18]. Conversely, the μ values for the C_{10} dimethyl dipeptide and the polymeric C_{10} dipeptide phase are somewhat higher, when compared to the reported value of 3.22 μmol m^{-2} for the C_{14} urea phases [19].

The new C_{10} dipeptide stationary phases were prepared by a one step surface modification in which the functional group was built into the silane. A single surface reaction with the silane yields only one possible ligand structure with no possibility of anion exchange functionality. The absence of anion exchange groups is an important advance with these new stationary phases. Fig. 2 compares a one- and two-step modified phase using a pH 3 buffered mobile phase for the separation of maleic acid, toluamide, and chlorpheniramine. Under these conditions, the analytes were negatively charged, neutral, and positively charged, respectively. For the C_{10} dipeptide phases (one step modification), all three analytes were eluted with short retention times and good peak shapes. For the Supelcosil ABZ plus column (two step modification), the neutral and positively charged analytes were eluted in a similar fashion, but negatively charged maleic acid was strongly retained with poor peak shape. We attributed the strong retention of maleic acid to ion exchange retention caused by residual amine groups on the surface. Thus, we recommend one step materials over the older two step materials for separations of negatively charged analytes.

3.2. Chromatographic evaluations

3.2.1. Column performance test

The first chromatographic evaluation was performed using a standard test mixture composed of uracil, acetylphenone, methylbenzoate, toluene and naphthalene using isocratic elution with a acetonitrile–water (60:40, v/v) mobile phase. All three columns tested demonstrate good separation of the test mixture. The C_{10} diisopropyl dipeptide phase shows a short retention indicating a lower hydrophobicity due to the lower surface coverage of 2.06 μmol m^{-2}. Retention factor, k, plate number per meter, N/m, and tailing factor at 5%, T_{x}, were calculated for each component. For naphthalene, the N/m values were 104,300, 110,360, and 87,000 and the tailing factors were 1.03, 1.02, and 1.05 for the polymeric C_{10} dipeptide phase, C_{10} dimethyl dipeptide phase, and C_{10} diisopropyl dipeptide phase, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Phase</th>
<th>Batch</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>μ (μmol m^{-2})</th>
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<tr>
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<td>21.49</td>
<td>3.62</td>
<td>3.64</td>
<td>3.63</td>
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<td></td>
<td>III</td>
<td>21.72</td>
<td>3.65</td>
<td>3.67</td>
<td>3.71</td>
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<tr>
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<td>I</td>
<td>22.76</td>
<td>3.82</td>
<td>3.77</td>
<td>3.78</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>22.59</td>
<td>3.81</td>
<td>3.79</td>
<td>3.75</td>
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<td>III</td>
<td>22.43</td>
<td>3.79</td>
<td>3.61</td>
<td>3.71</td>
</tr>
<tr>
<td>C_{10} diisopropyl dipeptide</td>
<td>I</td>
<td>17.16</td>
<td>3.03</td>
<td>1.72</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>17.02</td>
<td>3.11</td>
<td>1.69</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>17.34</td>
<td>3.09</td>
<td>1.74</td>
<td>2.10</td>
</tr>
</tbody>
</table>

*The mean ligand surface concentration of the polymeric C_{10} dipeptide phase, C_{10} dimethyl dipeptide phase and C_{10} diisopropyl dipeptide phase is 3.63, 3.75, and 2.07 μmol m^{-2}, respectively.*
ethylbenzene and toluene indicates that the stationary phase has capacity for the separation of compounds that differ only by a methylene group. The elution for \(N,N\)-DMA, with a symmetric peak before toluene and ethylbenzene, is another indication for the lower silanophilic interactions [22].

The retention factors for phenol are significantly higher on the new C_{10} dipeptide phases, due to a higher number of possible hydrogen bonds between the analyte and the polar group during the chromatographic separation. Such behavior has already been observed for other phenolic compounds on phases containing embedded carbamate groups and this enhanced selectivity is one of the features that differentiates packings with a polar group [23].

The polar dipeptide groups partially shield or minimize the effects caused by the residual silanol groups. One possible explanation is the presence of a hydrogen bonded layer of water molecules in the underlying surface, making the acidic residual silanol groups less accessible to interact with the basic analytes. Another possibility is that hydrogen bonding of the polar groups to the residual silanols masks their undesirable effects during the chromatographic process [24].

### 3.2.3. Tanaka test

The separation of two homologous alkyl benzenes, the non-planar and planar polyaromatic hydrocarbons, and caffeine and phenol, was performed on the polymeric C_{10} dipeptide phase with good efficiencies and peak shapes. For an easier comparison, the chromatographic parameters, obtained for the separation on both C_{18} and the polymeric C_{10} dipeptide phases, are summarized in Table 2. The shape selectivity, \(\alpha_{T/O}\), which gives information about functionality of the silyl reagent, and the methylene selectivity, \(\alpha_{CH_2}\), which gives information about the ability of a phase to distinguish between two compounds based upon a single methylene unit substitution, and is determined by injecting a series of \(n\)-alkylbenzene homologues, were calculated. A methylene selectivity of 1.52, 1.43, and 1.47 and a shape selectivity of 1.26, 1.59, and 1.35 were obtained for C_{18}, the polymeric C_{10} dipeptide phase, and the C_{10} dimethyl dipeptide phase. The \(\alpha_{T/O}\) value of 1.35 for the C_{10} dimethyl dipeptide phase is lower. It is agreed that the \(\alpha_{T/O}\) value is generally higher for “polymeric” phases (i.e., those prepared by silanization with di- or trifunctional silanes), and phases with longer alkyl chain lengths. The presence of a polar dipeptide group may also play a significant role in improving shape selectivity as a consequence of the \(\pi\) interactions between the polar group (i.e., carbonyl function) and the \(\pi-\pi\) active moieties on the less hindered planar triphenylene. This phenomenon was also observed for embedded polar phases with amide and carbamate groups [25] and polar endcapped phases with amine groups [26] as well as for alkylthiol and naphthalimide stationary phases [27]. The hydrogen bonding capacity, \(\alpha_{CIP}\), is a good measure of the number of available silanols and the degree of endcapping. It was calculated from the separation of caffeine and phenol, obtaining a value of 0.15 for the polymeric C_{10} dipeptide phase, which is much lower than the

<table>
<thead>
<tr>
<th>Compound</th>
<th>(C_{18})</th>
<th>Polymeric C_{10} dipeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k)</td>
<td>(N/m)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.12</td>
<td>33,920</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.46</td>
<td>52,010</td>
</tr>
<tr>
<td>Butylbenzene</td>
<td>8.15</td>
<td>102,520</td>
</tr>
<tr>
<td>Amylbenzene</td>
<td>12.38</td>
<td>87,210</td>
</tr>
<tr>
<td>(o)-Terphenyl</td>
<td>9.21</td>
<td>104,300</td>
</tr>
<tr>
<td>Triphenylene</td>
<td>11.58</td>
<td>104,500</td>
</tr>
</tbody>
</table>

### 3.2.2. Engelhardt test

The separation of the Engelhardt test mixture, containing thiourea (20 mg L\(^{-1}\)), aniline (40 mg L\(^{-1}\)), phenol (200 mg L\(^{-1}\)), \(p\)-, \(m\)-, \(o\)-toluidine (20 mg L\(^{-1}\)), \(N,N\)-dimethylaniline (20 mg L\(^{-1}\)), ethylbenzoate (200 mg L\(^{-1}\)), toluene, and ethylbenzene (1500 mg L\(^{-1}\)), was performed on the C_{10} dimethyl dipeptide column using methanol–water (55:45, v/v) as mobile phase at 298 K. \(p\)-, \(m\)-, and \(o\)-toluidines elute as a single peak, and aniline is eluted before phenol. This clearly indicates the absence of silanol activity in the stationary phase. Lower tailing factors for basic probes, aniline and \(N,N\)-dimethylaniline, and higher \(N/m\) values of 89,620 and 86,220 for the hydrophobic probes, toluene and ethylbenzene, were observed. The relative retention between phenol and toluene \(\alpha_{RT}\) was calculated, obtaining a value of 0.21. This value is similar to those obtained by Engelhardt et al. [20] for amide and carbamate polar reversed phases. This behavior is attributed to the hydrogen bonding contribution of the polar dipeptide group close to the silica surface. According to Engelhardt et al. [21] the separation factor, \(\alpha_{RT}\), between ethylbenzene and toluene, describes the hydrophobic selectivity of the column, while peak symmetry and retention times for the basic solutes in methanol:water provide information about its residual silanol activity. The separation factor between ethylbenzene and toluene is \(\alpha_{RT} = 1.79\) and that between toluene and \(N,N\)-dimethylaniline is \(\alpha_{RT/DMA} = 1.51\). Both separation factors show that the embedded dipeptide polar groups, followed by endcapping, reduced silanol interactions and minimized contact between residual groups and basic solutes. The value of the separation factor between

![Fig. 2. Chromatographic separation of acid, neutral, and base mixture at low pH on (a) C_{10} dimethyl dipeptide phase, (b) Supelcosil ABZ plus. Chromatographic conditions: mobile phase, MeOH:20 mM phosphate buffer (pH 3.0) = 20:80; flow rate, 1 mL min\(^{-1}\); detection, UV 254 nm. Peaks: 1= maleic acid; 2= toluamide; 3= chloropheniramine.](image-url)
value of 0.26 obtained for the C18 phase, due to the presence of the embedded polar dipeptide groups.

3.2.4. Neue test

To measure the hydrophobic, silanophilic and ion exchange properties of silica based reversed phase packings, Neue et al. [28] developed a method to test packing materials that employs a mixture of neutral, acidic and basic compounds under neutral mobile phase conditions. The shielding properties of the dipeptide groups, to minimize the undesirable interactions with unwanted surface silanols in the C10 dipeptide stationary phases, were evaluated using this test, containing uracil as a marker for column dead volume, naphthalene and acenaphthene as hydrophobic marker, dipropyl phthalate and butyl paraben as polar solutes, and propranolol and amitriptyline as basic probes. The separation of this mixture was performed on the polymeric C10 dipeptide and the C10 dimethyl dipeptide columns with good peak shapes and high efficiency. The same performance was not achieved on the C10 diisopropyl dipeptide phase. Under the same conditions, propranolol co-eluted with dipropyl phthalate and much higher tailing were observed for propranolol and amitriptyline. The tailing factors were 1.23 and 1.21 for propranolol and 1.26 and 1.24 for amitriptyline on the polymeric C10 dipeptide and C10 dimethyl dipeptide stationary phases that suppress ion exchange interactions due to reduced access to residual silanol groups. By comparing the k values, all compounds are less retained in the C10 diisopropyl dipeptide stationary phase. According to these results, we may conclude that the C10 diisopropyl dipeptide stationary phase is not as useful as the polymeric C10 dipeptide and C10 dimethyl dipeptide stationary phases for the separation of polar and basic compounds, mainly at neutral pH. One possible explanation for the observed decrease in the retention of almost all compounds and the higher tailing factors for the basic compounds is the lower surface coverage obtained for this phase with the sterically protecting diisopropyl groups. The main goal of the use of the sterically protected diisopropyl dipeptide phase was to enhance the hydrolytic stability of the silica support, which may be reduced by the presence of the embedded polar groups. However, from the results presented, the use of the C10 diisopropyl dipeptide stationary phase may be disadvantageous due to the dependence of retention and peak shape, especially for basic compounds, on the surface coverage of the stationary phase. The inherent ability of the dipeptide groups to minimize tailing for basic compounds seems to be affected by the lower concentration of these organic groups on the silica surface. On the other hand, promising results were observed for the C10 dimethyl dipeptide stationary phase, especially when compared with the polymeric C10 dipeptide phase, based on the same silica support. The tailing factors for propranolol and amitriptyline were lower for the C10 dimethyl dipeptide phase. One possible explanation for this better performance is the advantage of using monofunctional silane in the modification process, thereby avoiding extra methoxy groups which may produce more residual silanols on the silica surface during hydrolysis. Another possibility is the higher surface concentration obtained for the C10 dimethyl dipeptide stationary phase. The tailing factors observed for propranolol and amitriptyline with the C10 dimethyl dipeptide stationary phase were also lower when compared with the traditional C18 bonded phase.

3.2.5. Base deactivation properties

Pyridine has often been used to evaluate the adsorptive activity of stationary phases because it interacts more strongly with the residual silanol groups than aniline and its derivatives [4]. Phenol was used as the reference. The relative retention value ($k_{phenol/phen}$) was used for the evaluation of the effectiveness of endcapping. The C10 dipeptide and the Waters SymmetryShield RP18 columns were tested by separating a mixture of pyridine and phenol using acetonitrile–water (1:1, v/v) as the mobile phase. The asymmetry factors of pyridine were 1.45, 1.09, and 1.05 for the Waters SymmetryShield RP18, polymeric C10 dipeptide, and the C10 dimethyl dipeptide columns. The new C10 dipeptide columns demonstrated a higher separation factor of pyridine and phenol and a lower asymmetry factor of pyridine than the Waters SymmetryShield RP18 column.

3.2.6. Stability studies

An acidic or alkaline mobile phase was continuously passed through columns for 60 days at a flow rate of 1 mL/min, and the performance of the columns was examined periodically. The acidic mobile phase used was acetonitrile:1% TFA in water (pH 1.5) (1:1, v/v); the alkaline mobile phase was acetonitrile:20 mmol phosphate buffer (pH 11) (1:1, v/v). The stability of the ligands, the base silica, the endcapping groups, and the bonded dipeptide groups against hydrolysis were evaluated using the retention time ($t_R$) of benzene, the retention factor ($k$) of benzene, and asymmetry ($A_k$) of pyridine and benzene, respectively. The columns were tested by making injections of a four component test mixture using the pH 7.0 mobile phase. The results of chromatographic data provide conclusive evidence of the stability of the C10 diisopropyl dipeptide phase under stringent conditions. After 85 L of mobile phase had passed through the columns, the packings showed less than 2% change in the retention time of benzene at pH 1.5, and less than 5% change in the retention time of benzene at pH 11 (Fig. 3). These results revealed that the new C10 dipeptide stationary phases inhibited the dissolution of the base silica and that the dipeptide group on these stationary phases was not hydrolyzed under acidic mobile phase conditions.

3.3. Effects of mobile phase pH on selectivity

Because most pharmaceutical analytes are ionic or ionizable, varying the mobile phase pH is a powerful method development tool in reversed phase chromatography. For ionizable analytes, pH changes induce larger shifts in selectivity than traditional solvent changes. Acids are most retained at pH values below their pKₐ, at which they are predominantly in the uncharged protonated form. Conversely, bases are most retained at pH values above their pKₐ, at which they are predominantly in the uncharged unprotonated form. In general, retention is strongly dependent upon pH only within ±2 pH units of pKₐ. For a typical base with a pKₐ of 9, retention shifts occur over the pH range of 7–11. To access this full range, it is essential to use a column that is stable over a wide pH range. The new dipeptide functionalized silica stationary phases described here can be used from pH 1.5 to pH 11 (Fig. 3). This broad pH range allows great flexibility in HPLC method development.

To illustrate the dramatic shift in retention and selectivity possibly by varying mobile phase pH, we separated a mixture of acidic, basic, and neutral analytes on the C10 dimethyl dipeptide column using four mobile phase pH values (pH 2.5, 5.0, 8.0 and 10.0) (Fig. 4). At low pH values, the neutral analyte acetaminophen was eluted first, followed by the basic lidocaine, doxepin, imipramine, and nortriptyline with pKₐ values ranging from 7.9 to 9.7, and the acidic ibuprofen (pKₐ 4.4). At elevated pH values, the neutral and acidic analytes are eluted first, followed by the bases. The shoulder observed for peak 3 at pH 8.0 is caused by partial separation of the geometric isomers of doxepin. For this particular mixture, we obtained the best separation at pH 5.0.

It is interesting to note that the retention factor of the neutral acetaminophen (peak 1) at pH 10 is only half as large as that observed at a lower pH. In fact, acetaminophen is a weak acid (pKₐ 9.5) that is ionized at pH 10. These selectivity shifts may be advantageously applied in method development.
Fig. 3. Stability test on the C₁₀ dimethyl dipeptide phase at (a) pH 1.5 and (b) pH 11. pH 1.5 flush solution, MeCN: H₂O + 1% TFA (pH 1.5), 1:1; pH 11 flush solution, MeCN: 20 mM phosphate buffer (pH 11), 1:1. Chromatographic conditions: mobile phase, MeCN: 20 mM phosphate buffer (pH 7), 60:40; flow rate, 1 mL/min; detection, UV 254 nm. Peaks: 1 = uracil; 2 = pyridine; 3 = phenol; 4 = benzene.

Fig. 4. Separation of a mixture of acidic, basic, and neutral analytes on the C₁₀ dimethyl dipeptide column at (a) pH 2.5, (b) pH 5.0, (c) pH 8.0, and (d) pH 10. Chromatographic conditions: mobile phase, MeCN: 20 mM buffer, 30:70; flow rate, 1 mL/min⁻¹; detection, UV 220 nm. Peaks: 1 = acetaminophen; 2 = lidocaine; 3 = doxepin; 4 = imipramine; 5 = nortriptyline; 6 = ibuprofen.
3.4. Applicability of the new stationary phases

3.4.1. Separation of polar compounds in highly aqueous mobile phase conditions

Polar modified phases may wet more easily for various reasons, such as the hydrogen bonding ability of an embedded functional group with water that could drop the contact angle between the surface and water to less than 90°, at which water could penetrate the porous surface freely [29]. To probe the difference between alkyl and polar modified phases, we measured retention factor stability in 90, 95 and 100% aqueous mobile phase using a stop flow test. During this test, when the flow was stopped and the pressure was released from the column, we found that the mobile phase extruded from the pores of the purely alkyl phases due to poor wettability by the mostly aqueous mobile phases. After restarting the flow, we observed that the mobile phase remained outside of the pores, and the particle's accessible surface area was greatly diminished. As a result, the percentage decrease in retention factor for sulfacetamide approached 100% for the alkyl phases in changing from 90:10 (v/v) water–methanol to 100% water (Fig. 5). The C10 dipeptide phases were minimally affected across the three mobile phases, most likely because the aqueous mobile phase stayed within the pores, held by hydrogen bonding even without pressure on the column.

The separation of polar compounds under highly aqueous mobile phase conditions is not reproducible with conventional reversed phase materials. A proprietary derivatization procedure enables the C10 dipeptide phases to be penetrated by water without losing their hydrophobic property. The separations of water–soluble vitamins (Fig. 6) and nucleotides (Fig. 7) illustrate
the features of the C10 dipeptide phases in improving selectivity and peak shape and optimizing retention.

3.4.2. Separation of β-blockers under different pH conditions

The new C10 dipeptide phases demonstrate differences in selectivity and better separation characteristics for basic compounds compared to commercially available polar-modified phases. Fig. 8 demonstrates the separation of highly basic β-blocker drugs on the C10 dimethyl dipeptide phase, providing unique selectivity, high efficiency, and symmetrical peak shapes. The perfect resolution between propranolol and alprenolol was achieved only on the C10 dimethyl dipeptide column, and the separation of these two compounds was not possible on the Waters SymmetryShield RP18 and the Agilent ZORBAX Bonus-RP columns using these mobile phase conditions.

3.4.3. Separation of caffeine metabolites

Caffeine, 1,3,7-trimethylxanthine, is used world-wide as a drug and has been applied as a probe for the assessment of the phenotype of several liver biotransformation enzymes. In the human body, thirteen different metabolites of caffeine have been identified. These polar compounds are extremely difficult to retain and separate with reversed-phase chromatography. The separation of nine caffeine metabolites, each a geostereoisomer of similar structure (Fig. 9), on the C10 dimethyl dipeptide column, demonstrates the resolving power for positional isomers. The C10 dimethyl dipeptide column completes this separation in less than 13 min with remarkable selectivity and provides a noticeable reduction in retention time as well as substantial improvement in peak shape. Superior base-line resolution and outstanding peak symmetry of these compounds are observed on the C10 dimethyl dipeptide column whereas the commercially available columns exhibit coelution, incomplete resolution, and peak tailing (Fig. 10).

3.4.4. Separation of a mixture of tricyclic antidepressants and benzodiazepines

Tricyclic antidepressants are used to treat depression. They are also used to treat migraine, panic disorder, obsessive com-

![Fig. 8. Chromatographic separation of β-blockers at high pH on (a) the C10 dimethyl dipeptide phase, (b) the Waters SymmetryShield RP18, and (c) the Agilent ZORBAX Bonus-RP. Chromatographic conditions: mobile phase, MeOH:5 mM NH₄ HCO₃ (pH 10), 70:30; flow rate, 1 mL min⁻¹; detection, UV 220 nm. Peaks: 1 = pindolol; 2 = metoprolol; 3 = bisoprolol; 4 = propranolol; 5 = alprenolol.](image)

![Fig. 9. The structures of nine caffeine metabolites.](image)
pulsive disorder, recurrent headaches, and some forms of pain. Benzodiazepines belong to a group of substances known for their sedative, hypnotic, and anticonvulsant properties and are prescribed for therapeutic treatment of anxiety, sleep disorders, and convulsive attacks, and are sometimes used concomitantly with other classes of compounds, such as alcohol or antidepressants. Benzodiazepines, antidepressants, and their combinations are primary medications used today for panic disorder. There are several chromatographic methods for analysis of tricyclic antidepressants and benzodiazepines, among them gas chromatography using electron capture detection (GC–ECD), nitrogen–phosphorus detection (GC–NPD) and, more recently, mass spectrometric detection (GC–MS). Nevertheless, gas chromatography requires derivatization to provide thermostability to these drugs [30]. Here, a mixture of several members of these two classes of compounds, nitrozeapam, nordoxepin, alprazolam, diazepam, oxazepam, triazolam, nortriptyline, clonazepam, and trimipramine, was tested for preliminary applications in high-performance liquid chromatography. This mixture was eluted using the C10 dimethyl dipeptide column and the Waters SymmetryShield RP18 as well as the Agilent ZORBAX Bonus-RP columns for comparison (Fig. 11). The tricyclic antidepressants and benzodiazepines were separated with symmetric peaks and good resolution on the new C10 dimethyl dipeptide column, in contrast to the Waters SymmetryShield RP18 and the Agilent ZORBAX Bonus-RP columns where significant tailing and poor resolution were seen.
4. Conclusions

New C₁₀ dipeptide stationary phases with polymeric and sterically protecting dimethyl and diisopropyl groups were successfully prepared by a single step modification process. This methodology of preparing the organosilane with the desired substituents and then bonding it to the silica surface is advantageous over the two step modification process. The absence of a second reaction step allows obtaining a homogeneous composition attached to the silica surface. For the C₁₀ diisopropyl dipeptide phase, the compounds were not well separated and higher tailing factor values were observed, mainly for the basic compounds. These results, when compared to those obtained on the polymeric C₁₀ dipeptide phase and C₁₀ dimethyl dipeptide phase, may be attributed to a lower surface coverage which affects the overall chromatographic performance. Thus, the use of the C₁₀ dimethyl dipeptide silane is advantageous over the diisopropyl analog for preparation of such polar dipeptide phases.

Chromatographic parameters were evaluated using substances with different chemical properties. Characterization with hydrophobic, neutral, acidic, and basic probes was carried out using compounds from several different, well known test mixtures. The results show that there was an effective bonding of the new silane onto the silica surface and that the stationary phases were successfully endcapped. Characteristics such as hydrophobic selectivity, hydrogen bonding, shape selectivity, column stability, and absence of silanophilic interactions were acceptable. These new stationary phases, based on a C₁₀ dipeptide chemically bonded onto silica, exhibit good properties for applications in reversed phase for HPLC. Compared to conventional C₁₅ phases, these new dipeptide phases exhibit several advantages including different selectivity, improved peak shape for polar and basic compounds, stable retention in highly aqueous mobile phase conditions, and good hydrolytic stability. We presented examples showing the selectivity difference of these new dipeptide stationary phases compared to traditional C₁₅ columns. These properties make the new stationary phases a useful complement to conventional C₁₅ columns for a variety of HPLC applications.

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