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Synthesis, characterization, and application of a novel multifunctional stationary phase for hydrophilic interaction/reversed phase mixed-mode chromatography

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ABSTRACT

A novel multifunctional stationary phase based on silica gel was synthesised starting from L- isoleucine and 4-phenylbutylamine and evaluated as a hydrophilic interaction/reversed-phase mixed-mode stationary phase for high-performance liquid chromatography (HPLC). The prepared stationary phase was characterized by elemental analysis, infrared spectroscopy (IR), scanning electron microscopy (SEM), Brunauer, Emmett and Teller (BET) and solid-state ^{13}C nuclear magnetic resonance (NMR). The mechanisms involved in the chromatographic separation are multi-interaction, including hydrophobic, π-π, hydrogen-bonding, dipole-dipole and ion-dipole interactions. Based on these interactions, successful separation could be achieved among several aromatic compounds having different polarities under both hydrophilic interaction liquid chromatography (HILIC) and reversed phase (RP) condition. Nucleotides/nucleosides were separated in the HILIC mode. The effects of different separation conditions, such as pH value, mobile-phase content, column temperature, buffer concentration and flow rate, on the separation of nucleotides/nucleosides in HILIC mode were investigated. The seven nucleotides/nucleosides were separated within 22 min, while six of them were separated within 10 min by isocratic elution. To determine the influence of the new multifunctional stationary phase under the RP condition, a number of moderately and weakly polar and nonpolar compounds, such as 10 substituted anilines and eight substituted phenols were separated successfully under the RP condition within 14 and 15 min, respectively. Additionally, nine mixtures of polar/nonpolar test compounds were simultaneously separated within 19 min, while seven of them were separated within 12 min, under HILIC/RP mixed-mode conditions. Chromatographic parameters, such as the retention factor and peak asymmetry factor, were calculated for all of the analytes, while the theoretical plate number was calculated for analytes separated by isocratic elution. Compared to traditional C18 and commercial HILIC columns, the new stationary phase exhibited both HILIC and RPLC performance, and the scope of analyte separation was thus enlarged.

1. Introduction

The stationary phase is the main element of the HPLC system. The retention mechanisms, separation selectivity, and column efficiency mostly depend on the structure of the stationary phase [1]. Conventional HPLC stationary phases often possess a single interaction mechanism [2–6], while mixed-mode stationary phases [7–11] possess multiple interaction mechanisms between the stationary phases and analytes. At least two interactions that both contribute to the retention of the analytes should be present in the mixed-mode mechanism [12]. Compared with the conventional single-mode stationary phases, mixed-mode stationary phases possess the merits of high separation selectivity, remarkably high loading capacity, and high separation efficiency [13–18]. Till now, mixed-mode chromatography has been shown to be a versatile approach for the simultaneous separation of highly mixed classes of analytes from polar to nonpolar organic compounds [19–21].

A mixed-mode chromatographic stationary phase was first reported by Brown et al. [22] in 1986. The authors used a simple combination of ion-exchange and reversed-phase columns in series in mixed-mode chromatographic separation. In the same year, several materials designed for mixed-mode chromatographic stationary phases that could provide both anion exchange and hydrophobic interactions were

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Abbreviations: HPLC, High-Performance Liquid Chromatography; RPLC, Reversed-Phase Liquid Chromatography; HILIC, Hydrophilic Interaction Liquid Chromatography; DCC, Dicyclohexylcarbodiimide; HOBT, 1-Hydroxybenzotriazole hydrate; DCM, Dichloromethane; H, Hexane; EtOAc, Ethyl acetate; TFA, Trifluoroacetic acid; Boc, *tert*-Butoxycarbonyl; TLC, Thin-layer chromatography; THF, Tetrahydrofuran; AcOH, Acetic acid; ACN, Acetonitrile; DMSO, Dimethyl sulfoxide

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first proposed by Regnier et al. [23]. The authors prepared two materials. A polyamine was first adsorbed on the surface of silica beads, and then crosslinked with a multifunctional oxirane or acylated with a small anhydride molecule towards the amine groups, respectively. Columns packed with these materials could be operated in a mixed mode which was suitable for protein separation. From then on, more materials for mixed-mode chromatography were developed [1,24–26].

RPLC is the most-used HPLC mode for separation of moderately polar and weakly polar analytes, while HILIC is often used to separate strongly polar compounds. Thus, mixed-mode RPLC/HILIC can be used for the separation of both polar and nonpolar analytes, overcoming the deficiencies of RPLC and HILIC [27–46]. Some reported RPLC/HILIC stationary phase materials and their HPLC applications were given in related references in tabular form [26]. There is a continual increase in research in this area to find an ideal stationary phase and to investigate mixed-mode separation mechanisms. However, according to our best knowledge, usually either the polar or non-polar character of the previously reported mixed-mode stationary phases is very dominant. Thus, separations of very wide ranges of compounds from non-polar to highly polar have not been achieved. Therefore, there is a need for a stationary phase that several polar and non-polar groups are distributed more evenly.

In our previous works, several novel mixed-mode stationary phases were prepared [28–30]. The developed stationary phases displayed HILIC/RP mixed-mode retention characteristics and have been successfully used for separation of different groups of polar and nonpolar compounds. However, we have not yet obtained a sophisticated stationary phase that can cause separation on a very wide scale as we desire. Therefore, we are working diligently to identify the most ideal stationary phase.

In this study, a novel amide-functionalised silica-based multifunctional stationary phase was prepared starting from 4-phenylbutylamine and L-isoleucine as starting materials [Fig. 1]. The developed stationary phase possesses three amide groups as polar sites, as well as hydrophobic chains and an aromatic ring as nonpolar sites. It provided the equal distribution of hydrophilic and hydrophobic groups. Hence, this new stationary phase possesses both HILIC and RPLC dual retention mechanisms and was successfully used for separation of nucleotides/nucleosides, anilines, phenols and a mixture of polar-nonpolar aromatic compounds. The effect of some experimental conditions such as column temperature, mobile phase content, pH of mobile phase, flow rate and buffer concentration on the separations of nucleotides/nucleosides in HILIC mode was investigated. We named this new mixed-mode stationary phase "Ile-PBA-Mix" and it will be referred to by that name in this article.

2. Experimental

2.1. Reagents and materials

Spherical silica gel (Lichrospher Si 60, 5 µm, 60 Å, 700 m^2g^{-1}) was purchased from Merck. The solvents used for HPLC were of "HPLC grade" and were also purchased from Merck. All of the selected analytes (Fig. S1, Supplementary Materials) and all of the other reagents and solvents were purchased from Sigma-Aldrich or Merck. Empty HPLC columns were supplied from Kalite Sistem Merieux Nutrisciences company (Istanbul, Turkey). All of the reagents used in the synthesis were of "reagent grade" unless otherwise specified. The IR spectra were recorded on a Mattson 1000 FTIR spectrometer. Elemental analyses were performed with a Thermo Scientific FLASH 2000 instrument. Deionized water was purified using a Millipore Milli-Q water system. SEM images were obtained using a LEO EVO 40 model instrument (İnönü University, SEM Laboratory). Solid state ^{13}C NMR was recorded using a Bruker Superconducting FT.NMR Spectrometer Avance TM 300 MHz WB instrument (Middle East Technical University, Central laboratory, Solid state NMR laboratory). ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker DPX-400 High Performance Digital FT NMR Spectrometer (Dicle University, Faculty of Science, NMR Laboratory). Textural characteristics were determined by nitrogen adsorption at 67 K with an automatic adsorption instrument (Micromeritics, ASAP 2020). The BET surface area (S_{BET}), total pore volume (V_T) and pore-size distribution were determined from nitrogen adsorption-desorption data by using Quantachrome software.

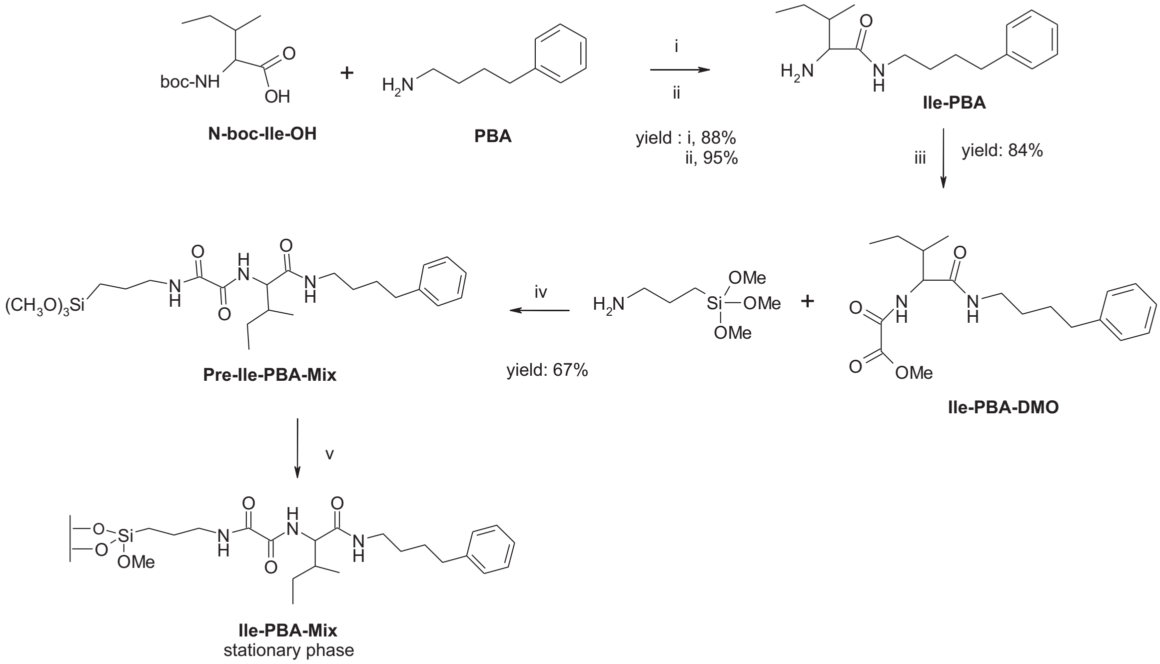

Fig. 1. *Conditions; i,* DCC, HOBT, dry CH_2Cl_2, 0 °C-then r.t., 24 h; *ii, TFA/* dry CH_2Cl_2, 0 °C-then r.t., 24 h; *iii, Dimethyloxalate/MeOH, r.t, 24 h.; iv,* MeOH, r.t., 24 h; *v,* Spherical silica gel, toluene-DMSO(7:1), 130 °C, 48 h.