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Separation of therapeutic peptides with cyclofructan and glycopeptide based columns in hydrophilic interaction liquid chromatography

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ABSTRACT

Three cyclofructan-based, two glycopeptide-based, and one zwitterionic column used in the HILIC mode were assessed within a graphical framework based on different functional characteristics contributing to selectivity. The characteristics of these six HILIC columns are put in the perspective of 33 columns evaluated previously. The isopropyl carbamate modified cyclofructan 6 (CF6) stationary phase, Larihc P, showed reduced component contributions for hydrophilicity and hydrogen bonding relative to the native cyclofructan 6 column (Frulic N). Both Frulic N and Larihc P exhibited cation exchange attributed primarily to deprotonation of residual unsubstituted silica with the greater exchange ascribed to the reduced loading of CF6 observed for Larihc P. The cyclofructan 6 column with a polymeric styrene divinylbenzene support (MCI GEL™ CRS100) showed distinct selectivities consistent with its decreased cation exchange attributable to its nonionic core. The Chirobiotic T, Chirobiotic V, and ZI-DPPS columns displayed hydrophilicity and ion exchange selectivities similar to other zwitterionic stationary phases. All of the more hydrophilic columns showed excellent separation for the four classes of therapeutic peptides investigated: microbial secondary metabolites used as immune suppressants, synthetic gonadotropin hormones, synthetic cyclic disulfide-linked hormone-regulating hormones, and non-ribosomally derived polycyclic antibiotics. Resolution provided by these columns and ZIC-HILIC is compared for each class of peptide. Frulic N is primarily suitable for use in the HILIC mode whereas Chirobiotic T, because of its increased efficiency and selectivity, can be useful in both HILIC and reverse phase modes. In some Chirobiotic T applications, addition of low levels of a strong additive (trifluoroacetic acid, formic acid, etc.) to the mobile phase can be beneficial. In these peptide analyses, a relative weakening of the often-dominant ionic interaction between analyte and residual charge on the stationary phase improved resolution and selectivity.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC), considered a variant of the normal phase modes of liquid chromatography [1–3], is characterized by the combination of a stationary phase with predominantly polar functionalities and a mobile phase that is a mixture of a predominantly aprotic polar organic solvent and water, often less than 30%. The preferred polar organic solvent is generally non-hydrogen bonding, most frequently acetonitrile. HILIC has been shown to be useful for analyses of an exceedingly wide range of polar analytes, including nucleic acids, nucleotides, and nucleosides; amino acids, peptides, lipo- and glyco-peptides, and proteins of both ribosomal and non-ribosomal origin; sugars, polysaccharides, and carbohydrates; simple and complex metabolites [1,4–13]. The versatility of HILIC is often exceptional [14–17]. HILIC separations are influenced by electrostatic interactions, hydrogen-bonding, dipole–dipole interaction, molecular shape selectivity reflecting steric interactions, and by hydrophobicity accompanied by the exclusion of water.

Several chemometric approaches have been explored to assess analyte interactions and evaluate columns [18–21]. Irgum and

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Table 1
Definitions and symbols used in the paper.

Symbol	Description	Definition
t_0	Retention time of dead volume	
t_i	Retention time of analyte i	
k(i)	Retention factor of analyte i	$k = (t_i/t_0) - 1 = (t_i - t_0)/t_0$
α	Retention factor ratio ($\alpha \geq 1$)	
α(A,B)	Retention factor ratio of compounds A and B (also selectivity or separation factor)	α(A,B) = k(A)/k(B) if k(A) > k(B) α(A,B) = k(B)/k(A) if k(B) > k(A)
α'(A,B)	Rectified selectivity of compounds A and B	α'(A,B) = k(A)/k(B) without constraint α'(A,B) = α(A,B) if k(A) > k(B) α'(A,B) = α(A,B)$^{-1}$ if k(B) > k(A)
$\lvert\mathbf{k}\rvert$	Length of vector k (or quasi-vector, if components are not quite orthogonal)	
$\lvert\mathbf{k_t}\rvert$	Length of vector k corresponding to all 5 analytes	$\lvert\mathbf{k_t}\rvert = (k(BMTA)^2 + k(cyt)^2 + k(ura)^2 + k(ado)^2 + (adi)^2)^{0.5}$
$\lvert\mathbf{k_{ni}}\rvert$	Length of vector k corresponding to 4 analytes, all of the polar analytes, but excluding that for cation exchange	$\lvert\mathbf{k_{ni}}\rvert = (k(cyt)^2 + k(ura)^2 + k(ado)^2 + (adi)^2)^{0.5}$

Note the log (α'(A,B)) is symmetric about 1.

coworkers [22] used principal component analysis to identify and segregate patterns of retention and selectivity of more than 20 analytes on more than 20 diverse commercial HILIC columns. One result of the analysis was the rank ordering of the selected probes; the other was the ordering of the columns. The two most diverse principal components captured 70–80% of the variation in response. Four groups were segregated, distinguished by the dominant functionality: positively charged and negatively charged, polar nonionic, and zwitterionic functionalities, the latter noted for their extensive solvation [12,22–24]. Positioning alternate HILIC columns within such a framework provides means of assessing dominant interactions with contrasting utility.

Lucy and coworkers simplified and streamlined the analysis [24,25]. These investigators selected only three retention factor ratios, two representing different nonionic types of interaction and one measuring cation exchange. A zwitterionic-sensitive ratio was not included [12,23,26]. These investigators evaluated the performance of the 21 columns investigated by Irgum as well as 8 additional HILIC columns and 4 reverse-phase columns and located them within three grids of retention factors ratios. In the study reported here, we utilized that approach to establish baseline attributes. The parameters and symbols used in the paper are summarized in Table 1.

Cyclofructans (CF's) have a chiral 18-crown-6 structure and consist of β-(2-1) linked D-fructofuranose units. Three columns with cyclofructan based stationary phases have been prepared in our laboratory and reported in the literature. Their different functionalities are summarized in Fig. S1 and include native cyclofructan 6 (CF6) (Frulic™ N) [27–29], an isopropyl carbamate modified cyclofructan 6 stationary phase (Larihc™ CF6-P) found to be excellent for chiral [30] as well as HILIC separations [31], and a 4-chloromethyl-styrene-divinylbenzene resin based CF6 column [32,33].

Two macrocyclic glycopeptide based stationary phases, Chirobiotic® T (Teicoplanin) and Chirobiotic® V (Vancomycin), were introduced in the 1990s [34–37]. They consist of aglycon portions of fused macrocyclic rings with a characteristic "basket" shape, yet contrasting stereogenic centers, sugar moieties, and hydrophobicity that contribute to distinctions in selectivity [38–41].

The sixth stationary phase assessed is zwitterionic, a phase with 3-P,P-diphenylphosphonium-propylsulfonate covalently bound to silica gel (ZI-DPPS) [42]. It possesses a negatively charged sulfonate group, a positively charged quaternary phosphonium group, and a diphenyl component imparting aromatic functionality. It showed improved retention, high peak efficiency and excellent peak symmetry in separation of β-blockers, nucleosides, and water-soluble vitamins [42,43].

Our study consists of two parts. First, small, probe molecule column characterization is used to assess the predominant modes of separation. This screening is based on selectivity, the α's (cf. Table 1), which is just the normal α that is not inverted to be greater than 1, something unnecessary when the ranking is based on a log scale. Secondly, we apply the recommendations from the probe analysis to columns and mobile phases for use in separation and analysis of therapeutic peptides. Four classes of therapeutic peptides were investigated, cyclosporin A and C, microbial secondary metabolites used as immune suppressants; buserelin, leuprorelin, goserelin, and gonadorelin, synthetic gonadotropin hormones; oxytocin, octreotide, and desmopressin, synthetic cyclic disulfide-linked hormone-regulating hormones; daptomycin, teicoplanin, and vancomycin, non-ribosomally derived polycyclic antibiotics utilized for penicillin- and other antibiotic-resistant infections. The structures of the five probe molecules and the different peptides from four peptide classes with quite different applications (cf. Section S4) are illustrated in Fig. 1. Estimated distribution coefficients (D) and isoelectric points of the peptides are provided in Table S1.

2. Experimental

2.1. Reagents

Goserelin acetate was purchased from US Biological (Salem, MA). The cyclosporins, the remaining gonadotropin salts, the cyclic hormones, the macrocyclic antibiotics and cytosine, uracil, benzyltrimethylammonium (BTMA), adenosine, adenine, ammonium acetate, acetic acid, trifluoroacetic acid (TFA), triethylamine (TEA), and formic acid were purchased from Sigma–Aldrich (St. Louis, MO). All of the gonadotropins were obtained as nonstoichiometric acetate salts. Acetonitrile of HPLC grade was obtained from EMD Millipore and water was purified using a Milli-Q Water Purification System (Millipore, Billerica, MA).

2.2. HPLC methods

All experiments were conducted on Agilent HPLC series 1200 systems (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump capable of virtually pulsatile free and high-pressure applications, an autosampler, and a multiwavelength UV–vis detector. For data acquisition and analysis, Agilent's Chemstation Software version Rev. B.01.03 was used in a Microsoft Windows XP environment. The injection volume was 5 μL and analytes were separated under isocratic conditions at 1 mL/min. Separations were carried out at room temperature. The dead time was determined from an abrupt change in baseline associated with unretained solvent.