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THE BEHAVIOR OF PROTEINS ON REVERSED-PHASE SUPPORTS DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PREDICTION OF PROTEIN RETENTION TIME ON *YMC* COLUMNS

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Protein molecules present more complex analytical challenges than conventional low molecular weight pharmaceutical compounds, and thus need powerful analytical approaches for the entire pharmaceutical development and quality control process. We used high-performance liquid chromatography to investigate the reversed-phase chromatographic behavior of eleven proteins (albumin, carbonic anhydrase, cytohrome c, L-glutamic dehydrogenase, enolase, α-lactoglobulin, Lysozyme, myoglobin and ribonuclease. By using a water/organic solvent/trifluoroacetic acid system the influence of experimental parameters was examined, and chromatographic results from two C4-chain-length supports were found to be comparable. The model enables prediction of initial conditions from two experimental data points for different types of reversed-phase columns with water-acetonitrile-TFA, water-methanol-TFA, and water-2-propanol-TFA mobile phases.

Key words: *high-performance liquid chromatography, mobile phase composition, protein, reversed-phase chromatography.*

INTRODUCTION

The RPLC separations of proteins can easily be tuned by changing the gradient slope, operating temperature, additives, pH, or organic modifiers [1-3]. The optimization of protein separations in RPLC has generally been achieved via the manipulation of the mobile phase with a given column; however, the use of different stationary phases, preferably with complementary selectivities, has also been successful [4].

The best approach to improve selectivity and thus resolution for peptides and proteins is to change the chemical nature or concentration of the organic modifier (e.g., acetonitrile, methanol, or isopropanol) and to select a suitable ion-pairing reagent [5].

The retention of peptides and proteins can be influenced by adding ion-pairing reagents to the mobile phase [6–8]. The ion-pairing reagents interact with the ionized groups of the proteins. Anionic counterions (e.g., hexanesulphonic acid, orthophosphoric acid, and trifluoroacetic acid) interact with the basic residues (i.e., arginine, lysine, and histidine) of a protein and with the protonated *N*-terminus. Cationic counterions (e.g., triethylammonium and

tetrabutylammonium) interact with ionized acidic residues (i.e., glutamic, aspartic or cysteic acid) and ionized free C-terminal carboxylic groups.

The major difference in method development for small analytes and large molecules such as proteins is related to the number of interactions (e.g., hydrophobic and ionic) that occur between the mobile and the stationary phase, the latter being strongly influenced by the conformations of proteins. Unfortunately, the changes in protein conformation that occur as the chromatographic conditions are varied are very complex and unpredictable [9].

Theoretical description of the mobile phase effect on the retention, selectivity and resolution of sample components enables prediction and optimization of gradient conditions and significantly reduces the number of experiments and time necessary for HPLC method development.

Therefore, method development that utilizes automated computer-assisted methods for predicting the retention properties on the basis of protein structure is not higly accurate. However, computer simulations of the retention behavior of peptides and proteins that is based on experimental chromatographic runs can still be a useful tool.

There are few instances found in the literature regarding applications of DryLab software for the computer-assisted method development of RPLC, for example, in cases of dialkylphthalate and nitroalkane separations [10, 11].

Retention times of small peptides were recorded and fitted to the linear relationship:

$$\mathrm{Tret_i} = \sum_{j=1}^{19} n_{ij} \cdot a_j + b_0 + \varepsilon_i \qquad (1)$$

where n_{ij} was the number of amino acid residues j in peptide i;

a_j – retention coefficient for residue j;

b_0 – retention coefficient for α-NH_2 and α-COOH terminal functions;

ε_i – independent errors which were assumed to be normally distributed, with the same variance.

Work by Gritti and Guiochon [12] marked significant progress in the investigation of protein band broadening by developing theoretically rigorous model for packed columns. Unfortunately, the model using numerical calculations was rather complicated for routine use.

The aim of this work was to explore the behavior of proteins on different YMC C4 columns with acetonitrile, methanol, and 2-propanol as organic modifiers during reversed-phase high-performance liquid chromatography. Protein retention time predictions in gradient elution mode were based on experimental chromatographic runs.

EXPERIMENTAL

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Methanol of HPLC grade, acetonitrile of HPLC grade,

2-propanol of HPLC grade and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Albumin (M_r~66500, No. 1), carbonic anhydrase (M_r~29000, No. 2), cytochrome c (M_r~12000, No. 3), L-glutamic dehydrogenase (M_r~290000, No. 4), enolase (M_r~67000, No. 5), α-lactoglobulin (M_r~18000, No. 6), lysozyme (M_r~14000, No. 7), myoglobin (M_r~18000, No. 8) and ribonuclease (M_r~14000, No. 9) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The HPLC analyses were performed using Agilent 1290 Infinity Quaternary LC System (Agilent Technologies, Santa Clara, CA, USA). This instrument includes a UV detector, a bin pump, a TCC column oven, and autosampler. Data acquisition, data handling and instrument control were performed by ChromSword Auto 4.0 Professional software. The columns used were YMC C4 (column 1; 150 × 4.6 mm I.D., 5 μm) and YMC Protein-RP (column 2; 75 × 2.1 mm I.D., 5 μm).

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile, methanol or 2-propanol (Appendix, Tables 1–6).

The flow rates were 1.0 mL/min for acetonitrile, 0.8 mL/min for methanol and 0.3 mL/min for 2-propanol. The column temperature was kept at 60 ^{o}C and the detection was at 210 nm wavelength. The injection volume was 20 μL. Proteins were dissolved in water at the concentration of 0.1 mg/mL.

RESULTS AND DISCUSION

The reversed-phase retention times of albumin, carbonic anhydrase, cytochrome c, L-glutamic dehydrogenase, enolase, α-lactoglobulin, lysozyme, myoglobin, and ribonuclease were determined using three different linear gradients, where eluent A was 0.1% aqueous TFA and the eluent B was 0.1% TFA in organic solvent. Under the conditions of reversed-phase liquid chromatography, the retention behavior of compounds can be normally described by the linear retention model (1):

$$\ln k' = \ln k_0' + a \cdot C , \qquad (2)$$

where k' is the retention factor;

C – the concentration of organic solvent in mobile phase [13].

The first step is to start with data from a small number of „well-chosen" experiments to predict retention for other conditons. In this case, the initial data sets are used as an input to build the retention models – the relationships between retention of compounds to be separated and concentration of organic solvent in the mobile phase. The linear solvent strength theory requires data from at least two runs to predict retention.

Different initial gradient experiments with linear gradient profiles were performed and retention time values for all studied compounds were measured. The results of experimental and predicted protein retention time are given in Tables 1 and 2.

Table 1. **Experimental and predicted retention time on YMC C4**[a]

Organic solvent	Acetonitrile			Methanol			2-Propanol		
Protein number	k_{exp}	k_{cal}	Dev.	k_{exp}	k_{cal}	Dev.	k_{exp}	k_{cal}	Dev.
1	22.76	22.77	0.01	13.04	13.06	0.02	17.17	17.13	0.04
2	23.77	23.88	0.11	16.66	16.69	0.03	21.63	21.60	0.03
3	14.27	14.27	0.00	10.80	10.75	0.05	15.07	15.01	0.06
4	6.70	6.72	0.02	15.86	15.87	0.01	12.63	12.67	0.04
5	7.73	7.79	0.06	10.02	10.09	0.07	13.54	13.54	0.00
6	24.35	24.35	0.00	16.78	16.77	0.01	19.62	19.64	0.02
7	20.32	20.32	0.00	14.06	14.07	0.01	17.60	17.57	0.03
8	22.79	22.73	0.06	18.80	18.76	0.04	20.59	20.55	0.04
9	13.47	13.47	0.00	7.87	7.88	0.01	13.71	13.79	0.08

Acetonitrile is the most commonly used organic solvent in HPLC. Isopropanol is often used for large or very hydrophobic proteins because of its superior elution strength, but its high viscosity presents a drawback. Methanol is also often used and preferred in process purifications and to elute hydrophobic proteins [1]. The results given in Tables 1 and 2 show a good agreement between the predicted (calculated) and experimental values. The deviations between the experimental and predicted retention times were not more than 0.11 minutes.

Table 2. **Experimental and predicted retention time on YMC Protein-RP**

Organic solvent	Acetonitrile			Methanol			2-Propanol		
Protein number	k_{exp}	k_{cal}	Dev.	k_{exp}	k_{cal}	Dev.	k_{exp}	k_{cal}	Dev.
1	18.58	18.56	0.02	10.85	10.86	0.01	20.09	20.03	0.06
2	21.22	21.24	0.02	11.98	11.97	0.01	23.04	23.05	0.01
3	14.13	14.07	0.06	7.40	7.42	0.02	16.40	16.33	0.07
4	9.17	9.17	0.00	12.82	12.83	0.01	15.44	15.45	0.01
5	7.06	7.09	0.03	14.68	14.69	0.01	16.68	16.74	0.06
6	18.21	18.22	0.01	11.45	11.42	0.03	20.81	20.84	0.03
7	18.46	18.46	0.00	10.04	10.03	0.01	19.36	19.38	0.02
8	20.67	20.70	0.03	14.84	14.83	0.01	22.32	22.33	0.01
9	11.35	11.25	0.10	11.53	11.52	0.01	15.53	15.64	0.11

[a] exp – experimental protein retention value
[b] cal – calculated (predicted) protein retention value

Such deviations appeared quite reasonable in practice for the prediction of retention during search for optimal gradient profile. The predicted elution order of the studied compounds corresponded to the experiment, as the predicted retention times were practically identical to the experimental values with correlation coefficient of 0.998 (Fig. 1 and Table 3).

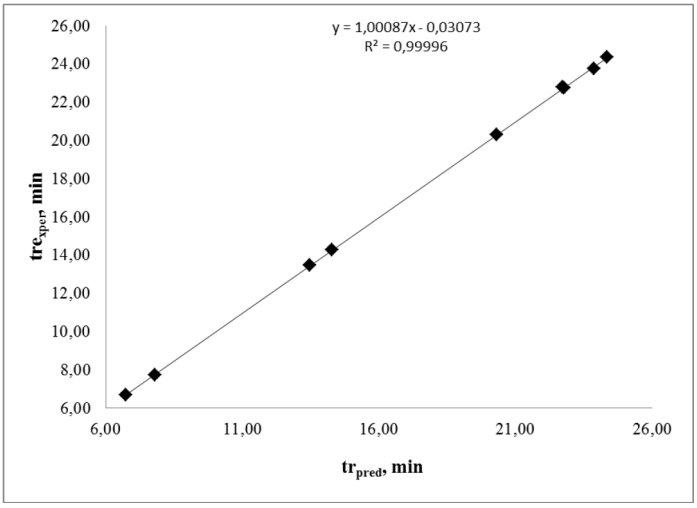

Fig. 1. Correlation of predicted and experimental retention time of proteins (1, 2, and 6–9) from the data of two experiments.

The linear retention model provided a good correlation between the experimental and predicted retention times of proteins with several different organic solvents (acetonitrile, methanol and 2-propanol) in mobile phase and on two types of columns (YMC C4 and YMC Protein-RP). The correlation coefficients are show in Table 3.

Table 3. **Correlation of predicted and experimental retention time of proteins [1, 2 and 6–9] from the data of two experiments.**

Organic solvent	Correlation, R^2	
	YMC C4	YMC Protein-RP
Acetonitrile	0.99996	0.99993
Methanol	0.99991	0.99996
2-Propanol	0.9998	0.9997

The linear retention model can be applied to proteins in a similar way as to the prediction of small retention. All of the used organic solvents (acetonitrile, methanol and 2-propanol) were suitable for such protein retention time prediction.

CONCLUSIONS

A simulated run can be carried out in less than a minute, saving more than 90% of the time required for an actual gradient-elution separation. At the same time, the computer can present results of a wider range of possible experiments.

The linear retention model has been applied to prediction of protein retention in gradient reversed-phase high-performance liquid chromatography. This model enabled prediction of initial conditions from two experimental data points for different types of reversed-phase columns with water-acetonitrile-TFA, water-methanol-TFA, and water-2-propanol-TFA mobile phases. The described method for prediction of retention can substantially reduce the time needed to find optimal conditions in gradient elution chromatography.

R E F E R E N C E S

1. Aguilar, M.I., Hearns, M.T.W. (1996). High resolution reversed phase high performance liquid chromatography of peptides and proteins. *Methods Enzymol.*, *270*, 3–26.
2. Mant, C.T., Hodges, R.S. (1997). Analysis of peptides by high performance liquid chromatography. *Methods Enzymol.*, *271,* 3–50.
3. Everley, R.A., Croley, T.R. Ultra-performance liquid chromatography/mass spectrometry of intact proteins. (2008). *J. Chromatogr. A*, *1192*, 239–247.
4. Fekete S., Veuthey, J.L., Guillarme D. (2012). New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: Theory and applications. *J. Pharm. and Biomed. Analysis*, *69*, 9–27.
5. Hancock, W., Bishop, C.A., Prestidge, R.L., Harding, D.R., Hearn, M.T.W. (1978). Reversed phase, high-pressure liquid chromatography of peptides and proteins with ion-pairing reagents. *Sci.*, *200*, 1168–1170.
6. Schuster, S.A., Wagner, B.M., Boyes, B.E., Kirkland, J.J. (2010). Wider pore superficially porous particles for peptide separations by HPLC. *J. Chromatogr. Sci.*, *48*, 566–571.
7. Shibue, M., Mant, C.T., Hodges, R.S. (2005). Effect of anionic ion-pairing reagent concentration (1–60 mM) on reversed-phase liquid chromatography elution behaviour of peptides. *J. Chromatogr. A*, *1080*, 58–67.
8. Getaz, D., Hariharan, S.B., Butte, A., Morbidelli, M. (2012). Modeling of ion-pairing effect in peptide reversed-phase chromatography. *J. Chromatogr. A*, *1249*, 92–102.
9. Staub, A., Guillarme, D., Schappler, J., Veuthey, J.L., Rudaz, S. (2011). Intact protein analysis in the biopharmaceutical field. *J. Pharm. and Biomed. Analysis*, *55*, 810–822.
10. Dolan, J.W., Snyder, L.R. (1987). Computer simulation as a means of developing an optimized reversed-phase gradient-elution separation. *J. Chromatogr.*, *24*, 261–276.
11. Dolan, J.W., Snyder, L.R., Djordjevic, N.M., Hill, D.W., Saunders, D.L. (1998). Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development: I. Application to 14 different samples using computer simulation. *J. Chromatogr. A*, *803*, 1–31.
12. Gritti, F., Guiochon, G. (2009). Peak compression factor of proteins. *J. Chromatogr. A*, *1216*, 6124–6133.
13. Roses, M., Subirats, X., Bosch, E. (2009). Retention models for ionizable compounds in reversed-phase liquid chromatography: Effect of variation of mobile phase composition and temperature. *J. Chromatogr. A,* 1216, 1756–1775.

OLBALTUMVIELU SORBCIJAS IZPĒTE AR APGRIEZTĀS FĀZES AUGSTI EFEKTĪVO ŠĶIDRUMA HROMATOGRĀFIJU UN IZDALĪŠANAS LAIKA PROGNOZĒŠANA *YMC* KOLONNĀM

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K O P S A V I L K U M S

Olbaltumvielu molekulas ir pēc uzbūves sarežģītākas nekā mazmolekulārie savienojumi, tāpēc ir nepieciešams pētīt risinājumus olbaltumvielu analizēšanai.Tika izmantota augsti efektīvā šķidruma hromatogrāfija, lai izpētītu vienpadsmit olbaltumvielu (albumīna, karboanhidrāzes, citohroma c, L-glutamāta dehidrogenāzes, enolāzes, α-laktoglobulīna, β-laktoglobulīna, lizocīma, mioglobīna un ribonukleāzes) uzvedību apgrieztās fāzes hromatogrāfijas apstākļos uz divām dažādām C4 kolonnām ar ūdens/organiskais šķīdinātāja/trifluoretiķskābes kustīgajām fāzēm.

Mūsu pārbaudītais teorētiskais modelis ļauj prognozēt izdalīšanās laikus, izmantojot divus eksperimentālo datu punktus uz dažāda veida apgrieztās fāzes kolonnām ar ūdens-acetonitrila-TFA, metanola-ūdens-TFA un ūdens-2-propanola-TFA kustīgām fāzēm.

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