Journal of Chromatography, 269 (1983) 96–102
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,071

Note

Use of fluorinated bonded phases in reversed-phase high-performance liquid chromatography of proteins

GENG XINDU* and PETER W. CARR*
Department of Chemistry, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)
(First received March 24th, 1983; revised manuscript received June 15th, 1983)

During the past several years there has been considerable interest in reversed-phase high-performance liquid chromatography (HPLC) based on the use of non-polar surfaces other than bound hydrocarbon ligands. Such materials include graphitized carbon[1], XAD-2[2], and most recently silica gel derivatized with fluorocarbon silanes[3–5]. Berendsen[3], Billiet[4] and Haas[5] indicate that retention of non-polar solutes on fluorocarbon bonded phases is considerably less than on comparable hydrocarbonaceous bonded phases under the same mobile phase conditions. Selectivity between various solutes is preserved. These characteristics are quite interesting and potentially useful for the development of reversed-phase separations of proteins by HPLC since one should be able to bring about elution of proteins with considerably less organic modifier in the mobile phase, *i.e.* weaker mobile phases. This should be beneficial from two points of view. First, lower concentrations of organic modifier are typically less protein denaturing. Second, removal of lower levels of organic modifier should be less arduous in preparative separations.

A third potential advantage is that fluorocarbon liquids are known to be much less denaturing and less adsorptive towards proteins than are hydrocarbonaceous materials. Also, certain fluorocarbon liquids are useful as blood substitutes and are therefore biocompatible[6]. We infer that when using fluorinated bonded phases, recoveries of proteins may be considerably improved relative to those obtained with hydrocarbonaceous reversed-phase ligands. In this note we report the first HPLC separation of proteins on highly fluorinated bonded phases.

EXPERIMENTAL

All work reported here was carried out on a chromatographic system comprised of an Altex-Beckman (Berkeley, CA, U.S.A.) Model 110 A pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 injection valve fitted with a 20-μl loop and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model LC-15 absorbance detector at a wavelength of 254 nm. The two packing materials used in this work were obtained from ES Industries (Marlton, NJ, U.S.A.). The conventional hydrocarbon column has a

* Honorary Visiting Scholar from Department of Chemistry, The Northwestern University, Xian, China.

0021-9673/83/$03.00

decyl dimethylchlorosilane bonded phase, and will be designated the HC-10 column. The fluorocarbon column has a (heptadecafluorodecyl)dimethylchlorosilane bonded phase and will be designated the FC-10 column. Both bonded phases were packed in similar tubes (5.0 cm × 4.6 mm) by a stirred slurry technique. The FC-10 was packed from 100% tetrachloromethane, whereas the HC-10 was packed from 100% methanol. The analytical columns were jacketed and thermostatted at the indicated temperatures by use of a circulating controller (Haake, Model F, Berlin, F.R.G.). Both columns were fully equilibrated with a new mobile phase by flushing with a minimum of 60 ml of that mobile phase.

The mobile phases used in this work were prepared using deionized water which was subjected to carbon filtration (Continental Demineralization System, Minneapolis, MN, U.S.A.). Isopropanol (HPLC grade) was obtained from Mallinckrodt (Paris, KY, U.S.A.). Buffers were prepared from potassium dihydrogen phosphate (Fisher, analytical-reagent grade) and trifluoroacetic acid (99% pure, Aldrich, Milwaukee, WI, U.S.A.). The aqueous buffers were prepared and adjusted to the final pH by addition of potassium hydroxide or phosphoric acid. The final solutions were prepared by mixing the buffer and appropriate quantity of isopropanol, and were filtered through a 0.2-μm membrane (Millipore, Bedford, MA, U.S.A.). As pointed out by Berendsen[3] the fluorocarbon columns are difficult to operate with mobile phases containing less than 40% methanol due to baseline instability. We have found that continuous purging of the mobile phase with helium[7] greatly alleviates the problem observed by Berendsen. In order to prevent loss of organic modifier from the solvent reservoir, it was necessary to pre-saturate the helium by passing it through a gas washing bottle containing the mobile phase of interest.

The proteins used in this work were: ribonuclease A and thyroglobulin (Pharmacia, Piscataway, NJ, U.S.A.), and bovine pancrease insulin, egg white lysozyme (grade I), horse heart cytochrome *c*, and bovine albumin (fraction V) (Sigma, St. Louis, MO, U.S.A.). With the exception of thyroglobulin, all proteins were dissolved in the same mobile phase as used for elution. Thyroglobulin was dissolved in isopropanol–0.05 *M* KH_2PO_4 (pH 1.5) buffer (15:85) and filtered through a 0.2-μm filter prior to use since it was not very soluble in all the mobile phases used in this work. The stock protein solution concentrations were all 1 mg/ml except for thyroglobulin, in which case a saturated solution was employed. In those cases in which a mixture of proteins was separated the following total amounts of protein were injected: ribonuclease A (2 μg), insulin (6 μg), cytochrome *c* (16 μg). For all work described here the flow-rate was 2.0 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows an isocratic separation of 3 proteins under identical mobile phase conditions. It is clear that retention of each protein is considerably less on the FC-10 column (Fig. 1B) than on the HC-10 column (Fig. 1A).

The effect of organic modifier on the retention of proteins on the FC-10 and HC-10 column is summarized in Table I. The capacity factors, k', were computed from the total retention volume based on the use of the apparent average elution volume of ^2H_2O and potassium dichromate to estimate the dead volume (see Table III).

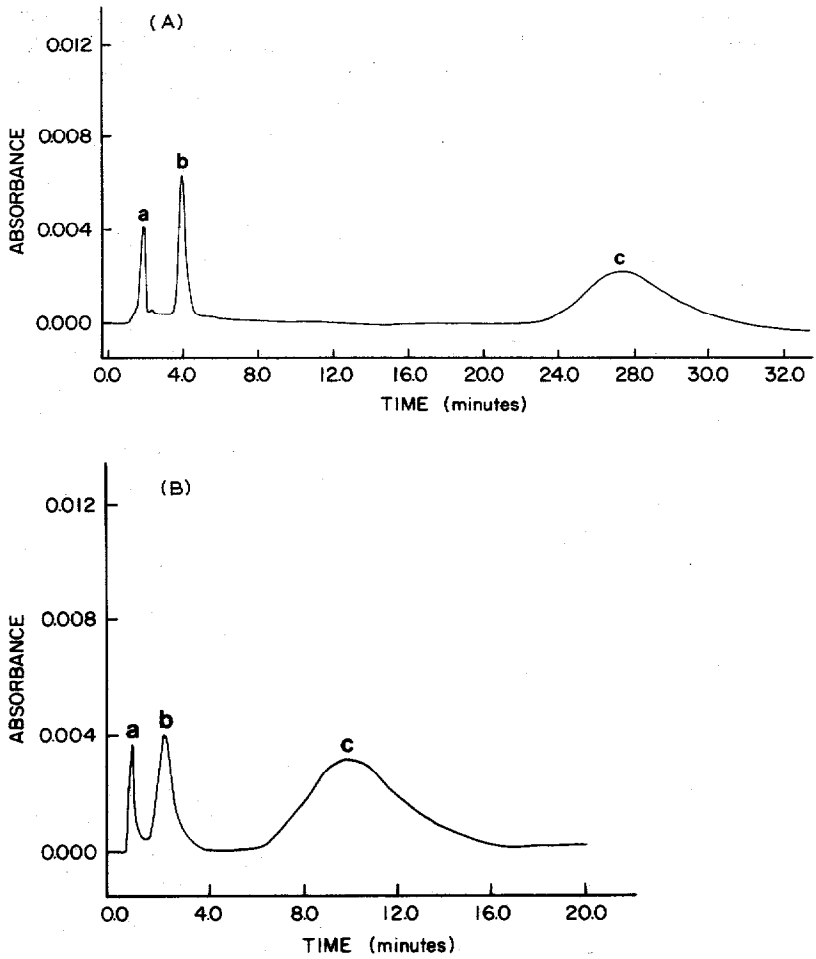

Fig. 1. Chromatogram of protein mixture. The sample proteins are: ribonuclease (a), insulin (b), and cytochrome *c* (c). Eluent in both cases is: isopropanol–0.05 *M* KH_2PO_4 (19:81), pH 3.00; temperature, 47°C. (A) HC-10 column; (B) FC-10 column.

The ratio of k' values for a given protein on the two column types is displayed in Table I as the quantity in brackets. No values are reported when k' values on both columns are less than 0.2 because the uncertainty in k' at low retention values is far too great to permit a valid comparison. In all cases for which k' is greater than about 0.5 on the fluorocarbon column, the hydrocarbon column has a larger k' value. It should be noted that the ratio of k' values increases towards unity as k' decreases, *i.e.* as the mobile phase is made stronger. Thus, in a given mobile phase, the decrease in retention is greatest for the more strongly retained protein. This is most clearly evident in comparing the retention of BSA in isopropanol–buffer (32:68). Consequently, it appears that the more hydrophobic the protein, the greater the effect of a change from HC-10 to FC-10.

The effect of column type on selectivity, *i.e.* the ratio of retentions of two

TABLE I

PROTEIN CAPACITY FACTORS, k', ON HC-10 AND FC-10

The solvents are the indicated mixture of isopropanol with 0.05 *M* KH_2PO_4, 1.0% trifluoroacetic acid, pH 3.00 buffer adjusted with KOH and H_3PO_4. (Temperature of analytical column is 47°C.) All quantities in parentheses are the ratio of k' on the FC-10 column to that on the HC-10 column. A blank entry (–) indicates that k' is greater than ≈60 and was not eluted until the solvent strength was increased.

Protein	*Column Type*	*Solvent composition (isopropanol–buffer)*						
		20:80	*22:78*	*24:76*	*26:74*	*28:72*	*30:70*	*32:68*
Ribonuclease	FC-10	6.55	1.87	0.91	0.37	<0.2	<0.2	<0.2
	HC-10	15.7	2.68	1.18	0.50	<0.2	<0.2	<0.2
		(0.42)	0.70)	(0.77)	(0.74)	–	–	–
Insulin	FC-10	12.94	3.92	1.90	1.02	0.63	0.46	0.40
	HC-10	47.4	9.26	4.13	1.81	0.80	0.43	0.37
		(0.27)	(0.42)	(0.46)	(0.56)	(0.78)	(1.06)	(1.08)
Lysozyme	FC-10	–	–	–	17.4*	4.65	1.80	1.06
	HC-10	–	–	–	26.73	13.39	2.70	1.43
		–	–	–	–	(0.347)	(0.67)	(0.74)
Cytochrome *c*	FC-10	–	–	–	22.2	3.98	1.18	0.65
	HC-10	–	–	–	51.8	5.95	1.04	0.48
		–	–	–	(0.43)	(0.67)	(1.13)	(1.35)
BSA	FC-10	–	–	–	–	–	25.0	2.46
	HC-10	–	–	–	–	–	–	16.6
		–	–	–	–	–	–	(0.15)
Thyroglobulin	FC-10	–	–	–	–	–	–	5.37
	–	–	–	–	–	–	–	–

* For this entry the mobile phase composition is 27.5% isopropanol.

proteins under the same conditions, is shown in Table II. It is clear that the selectivity is definitely greater on hydrocarbonaceous supports than on fluorocarbon supports. This is in direct contrast to the behavior of small solutes. However, in no case is the selectivity so poor as to cause a separation problem with the set of proteins tested in this work.

The last general factor which needs to be addressed is the comparative solvent strength scales for these two types of columns. In Fig. 2 ln k' values are plotted *vs.* the volume fraction of organic modifier. In contrast to what is observed in reversed-phase chromatography of small solutes, linearity in these plots exists over only a very narrow range in concentration of organic modifier ($<\sim$10%). This makes the comparison of relative solvent strength on the two columns quite difficult. Where linearity exists, *e.g.* in the case of ribonuclease from 22 to 26% isopropanol, the effect of solvent on retention appears to be very comparable for HC-10 and FC-10. For the other species it is evident that an increase in solvent strength accelerates the movement of a solute on the HC-10 column to a greater extent than on the FC-10 column. We infer that the differential solvent effect on the two columns must take place

TABLE II

COLUMN SELECTIVITY EFFECTS

Values in the table are the ratio of retention of the indicated pair of proteins on each column. The eluent is the indicated mixture of isopropanol and 0.05 *M* KH_2PO_4 buffer 1.0% trifluoroacetic acid. The buffer solution was adjusted to pH 3.0 with KOH and H_3PO_4.

Protein pair	*Column type*	*Solvent composition (isopropanol–buffer)*						
		20:80	*22:78*	*24:76*	*26:74*	*28:72*	*30:70*	*32:68*
Ribonuclease, insulin	FC-10	1.97	2.09	2.08	2.75	–	–	–
	HC-10	3.01	3.47	3.50	3.62	–	–	–
Insulin, lysozyme	FC-10	–	–	–	17.05	7.38	32.91	2.65
	HC-10	–	–	–		16.7	6.27	3.86
Cytochrome *c*, BSA	FC-10	–	–	–	–	–	21.1	3.78
	HC-10	–	–	–	–	–	–	34.5
BSA, thyroglobulin	FC-10	–	–	–	–	–	–	2.18
	HC-10							

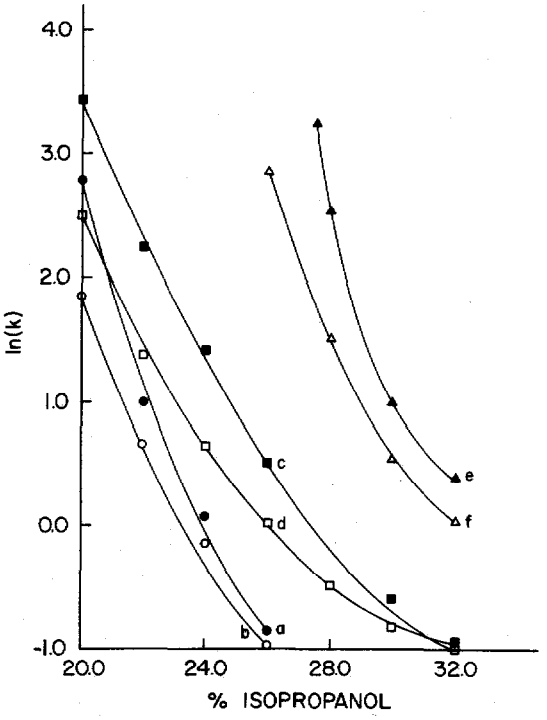

Fig. 2. Comparison of solvent strength on the hydrocarbon and fluorocarbon columns. All conditions are as in Table I. (a) ribonuclease on HC-10; (b) ribonuclease on FC-10; (c) insulin on HC-10; (d) insulin on FC-10; (e) lysozyme on HC.10; (f) lysozyme on FC-10.

TABLE III

EFFECT OF SOLVENT ON COLUMN DEAD VOLUME

Dead volume taken as solvent volume required to achieve peak maximum with the indicated solutes. The buffer in all cases is 0.05 *M* KH_2PO_4 + 1% trifluoroacetic acid. The buffer solution was adjusted to pH 3.0 with KOH and H_3PO_4.

Solvent (isopropanol–buffer)	*FC-10*			*HC-10*			*Ratio***
	^2H_2O	$K_2Cr_2O_7$	*Average**	^2H_2O	$K_2Cr_2O_7$	*Average**	
20:80	0.630	0.630	0.630	0.664	0.655	0.660	1.048
24:76	0.608	0.612	0.610	0.650	0.644	0.647	1.060
28:72	0.600	0.600	0.600	0.638	0.642	0.640	1.066
32:68	0.586	0.600	0.593	0.640	0.630	0.635	1.070

* Average of dead volume obtained with ^2H_2O and $K_2Cr_2O_7$.
** Ratio of the average dead volume on the HC-10 column to the FC-10 column.

through an interaction of the solvent with the stationary phase. This was substantiated by determining the dead volume at each mobile phase composition using ^2H_2O and potassium dichromate as dead volume markers. McCormick and Karger[8] have carried out a detailed study of the effect of solvent on the apparent dead volume. They showed that the dead volume was not a constant physical characteristic of the column but that it varied with mobile phase composition. As shown in Table III, the dead volume is not systematically higher for ^2H_2O than for potassium dichromate. We believe that at the low pH used in this work the silanol groups are totally protonated and therefore there is no charge exclusion of the dichromate anion. It is evident that the average dead volume decreases as organic modifier was added to the mobile phase, whereas the ratio of dead volumes for the HC-10 to FC-10 increased. Clearly there is a differential effect of solvent on these two stationary phases. Thus

TABLE IV

RELATIVE PROTEIN RECOVERY ON FC-10 AND HC-10

In all cases the mobile phase is comprised of isopropanol–0.05 *M* KH_2PO_4, 1% trifluoroacetic acid, pH 3.0 (32:68). Columns are operated at 47°C at 2 ml/min.

Protein	*Recovery*	
	HC-10	*FC-10*
Ribonuclease	83	103
Insulin	80	113
Lysozyme	98	109
Cytochrome *c*	87	103
BSA	—*	80
BSA	43**	95**
BSA	83***	102***

* No peak obtained.
** Mobile phase changed to isopropanol–buffer (33:67).
*** Mobile phase changed to isopropanol–buffer (34:66).

we must expect that the solvent strength scales on these two types of related columns will differ.

A major problem in all forms of protein chromatography is the incomplete recovery of sample. In order to verify that recovery characteristics of the fluorocarbon columns are acceptable, we estimated protein recoveries on the HC-10 and FC-10 columns under identical conditions. In each case the same amount of protein was injected into both columns. Recovery was computed as the ratio of peak area in the presence and absence of the analytical column. Such measurements are extremely difficult to carry out accurately. A direct comparison of recoveries on the two columns is presented in Table IV. Some values are in excess of 100% due to underestimation of the area of the very narrow tailed peaks obtained in the absence of the column. In all cases the recovery of protein on the FC-10 column exceeded that on the HC-10 column. It should be noted that reported recoveries of the very hydrophobic BSA[9] on hydrocarbonaceous reversed-phase columns is quite low. We were able to achieve nearly 80% recovery on FC-10.

These preliminary studies, based on a limited number of test proteins, indicate that fluorocarbon bonded phases may be generally useful in the reversed-phase separation of proteins. A detailed communication on the properties of fluorinated ligands is in preparation.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the 3M Company, St. Paul, Minnesota.

REFERENCES

1 H. Colin, C. Eon and G. Guiochon, *J. Chromatogr.*, 119 (1976) 41.
2 J. L. Robinson, W. J. Robinson, M. A. Marshall, O. B. Kenneth, J. Johnson and D. S. Salas, *J. Chromatogr.*, 189 (1980) 145.
3 G. E. Berendsen, K. A. Pikaart, L. de Galan and C. Olieman, *Anal. Chem.*, 52 (1980) 1990.
4 H. A. H. Billiet, P. J. Schoenmakers and L. de Galan, *J. Chromatogr.*, 218 (1981) 443.
5 A. Haas, J. Kohler and H. Hemetsberger, *Chromatographia*, 14 (1981) 341.
6 L. C. Clark, Jr., E. P. Wessler, M. L. Miller and S. Kaplan, *Microvascular Res.*, 8 (1974) 320.
7 S. R. Bakalyar, M. P. T. Bradley and R. Honganen, *J. Chromatogr.*, 158 (1978) 277.
8 R. M. McCormick and B. L. Karger, *Anal. Chem.*, 52 (1980) 2249.
9 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.