Journal of Chromatography, 512 (1990) 315–323
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1861

Tryptic fingerprinting on a poly(styrene–divinylbenzene) reversed-phase column

JOEL K. SWADESH
Polymer Laboratories, Inc., 160 Old Farm Road, Amherst, MA 01002 (U.S.A.)

ABSTRACT

The properties of poly(styrene–divinylbenzene) (PS–DVB) in gradient reversed-phase peptide separations have not been fully explored. Comparisons of selectivity and fraction capacity with silica alkyl bonded phases remain to be established. Investigation of the effects of gradient rate and flow-rate on separation are desired. The present work examines separations of synthetic peptides and of tryptic fragments of three species of cytochrome *c* and reduced, carboxymethylated human plasminogen. Fingerprinting is a means to localize the position of one or more variant amino acids within the sequence of a large polypeptide. Reproducibility of separation is a dominant issue, potentially affected by variability in pump performances. The susceptibility of the peptide fingerprint to changes in pump performance can be examined by systematic variation of the flow-rate and gradient rate. PS–DVB exhibited predictable separations equivalent to those of alkyl bonded phases. The selectivity parallelled that observed for bonded phases. Two observations made in the course of these studies may be of interest to the theory of reversed-phase liquid chromatography. First, the peak capacity was found to be a simple function of the gradient rate. Also, the resolution was observed to increase with increasing flow-rate in a separation of horse cytochrome *c* tryptic fragments at fixed gradient rate.

INTRODUCTION

There have been many elegant demonstrations of applications of peptide mapping by various separations techniques, particularly reversed-phased liquid chromatography[1–3]. Reversed-phase high-performance liquid chromatography (RP-HPLC) is presently perhaps the most widely used technique, combining high reproducibility and resolving power with low sample requirements and easy post-separation recovery. Poly(styrene–divinyl benzene) (PS–DVB) has become widely accepted as a high-performance reversed-phase material only recently[4–6], so many of its applications in separations remain to be demonstrated. PS–DVB is known to exhibit a number of properties relevant to ruggedness in fingerprinting, among which are pH stability and ease of cleaning. The present work examines PS–DVB selectivity and peak capacity, using as probes a series of synthetic peptides, tryptic digests of cytochrome *c* from three species, and the tryptic digest of human plasminogen.

0021-9673/90/$03.50

EXPERIMENTAL

Materials

The HPLC system consisted of two Model 364 pumps, a Model 50 HPLC Programmer, and a Model 87.00 UV detector equipped with a 3-mm 0.8-μl flow-cell (Knauer, Berlin, F.R.G.). High-pressure mixing was accomplished with a 10-μl Model TCMA 0120113T Visco-Jet mixer (Lee Co., Westbrook, CT, U.S.A.). Injection was performed with a Model 231 autoinjector (Gilson, Middleton, WI, U.S.A.) equipped with a 100-μl sample loop. Separations were accomplished on a 25 × 0.46 cm I.D. 100 Å PLRP-S column (Polymer Labs.), Church Stretton, U.K.). Solvents were degassed and maintained under a positive pressure of helium.

Oxalic acid, sodium iodoacetate, ammonium bicarbonate, cytochrome *c* species variants (horse type VI, tuna type XI and rabbit type XV) and the synthetic peptides (single-letter code for amino acids used) GFL, YY, YF, GYG, GLY, YGGFM, YG, YV, PY, GY, YA, YGGFL, YAGFM, YGGFLK, YGG, GGFM, FV, YL, YE, RVYIHPF and DRVYIHPF were obtained from Sigma (St. Louis, MO, U.S.A.). Benzoic acid was from Chem Service (West Chester, PA, U.S.A.). Trishydroxymethylaminomethane (Tris), dithiothreitol (DTT), disodium ethylenediaminetetraacetic acid (EDTA) and guanidine hydrochloride (GuHCl) were from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Acetonitrile, 2-propanol and trifluoroacetic acid were from Baker (Phillipsburg, NJ, U.S.A.). Dialysis membrane with a 3500 molecular weight cutoff was from Spectrum Medical (Los Angeles, CA, U.S.A.). Glu-plasminogen from human serum was from Serva (Westbury, NY, U.S.A.). Sequencing grade bovine pancreatic trypsin was from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Methods

Reversed-phase separations were conducted at a flow-rate of 1 ml/min unless otherwise specified. The aqueous mobile phase was prepared from 990 ml water, 10 ml 2-propanol and 1 ml trifluoroacetic acid. The organic mobile phase was 990 ml acetonitrile, 10 ml 2-propanol and 1 ml trifluoroacetic acid. For separations of the synthetic peptides, oxalic acid was added to the sample as an injection marker and benzoic acid was used as a gradient marker. About 5–25 μg of each synthetic peptide was injected.

A stock solution of 0.5 *M* Tris, pH 8.86 was prepared. Reduction of 10 mg plasminogen was performed at 37°C in 1 ml 100 m*M* Tris, 1 m*M* EDTA, 6.3 *M* GuHCl. Incubation with 4.9 mg DTT proceeded for 1 h. The reaction was quenched with 2.7 mg sodium iodoacetate in 210 μl water and allowed to stir 45 min. Then, the sample was dialyzed three times against 0.5 *M* ammonium bicarbonate (200 ml; 1 h) and once against 4 l of 0.1 *M* ammonium bicarbonate. The species of cytochrome *c* were also dialyzed into 0.1 *M* ammonium bicarbonate.

Trypsin digestion of 5 mg of plasminogen and 5 mg of each of the cytochrome *c* variants was performed at 37°C in 1.4 ml 0.1 *M* ammonium bicarbonate. Aliquots of 20 μg of trypsin (1 μg/μl) were added at 0, 1, 2, 4 and 8 h. The progress of the digestion was monitored by RP-HPLC to ensure completion. About 35 μg of trypsinized cytochrome *c* and 350 μg trypsinized plasminogen were injected in 100 μl. In all cases, chromatography was performed at ambient temperature (24 ± 2°C).

RESULTS AND DISCUSSION

Illustration of fingerprinting

Fig. 1 shows tryptic fingerprints of three species of cytochrome *c*. The peptides containing the protoporphyrin IX heme moiety were established by detection at 400 nm (data not shown) and are marked with an asterisk in the figure. Despite 85–95% homology between the species, the fingerprints are distinctive. Tryptic fingerprints of horse cytochrome *c* on several alkyl bonded phases are available in the literature[2].

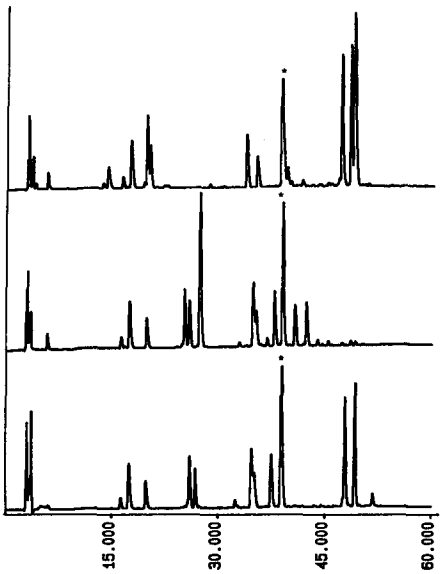

Fig. 1. Tryptic fingerprints of species of cytochrome *c*. Reversed-phase separation of tryptic fragments of about 35 μg tuna (upper panel), horse (middle panel) and rabbit (lower panel) cytochrome *c* on a 25 × 0.46 cm I.D., 5-μm, 100-Å PLRP-S column, using acetonitrile–water–0.1% trifluoracetic acid–2-propanol as the mobile phase. Gradient conditions were aqueous-organic mobile phase (95:5) (0–5 min) to aqueous–organic (60:40) (5–55 min), 1 ml/min. The ordinate is the absorbance at 220 nm, while the abscissa is retention in minutes. The peaks marked with an asteriks exhibited absorbance at 400 nm and were therefore assigned as peptides containing heme.

Selectivity

Fig. 2 is a graph of the peptide retention times observed on PLRP-S, shown on the *y*-axis, *versus* the retention times predicted by a theory developed for alkyl.bonded phases[7,8], shown on the *x*-axis as the sum of retention coefficient ($\sum R_c$) corrected for gradient rate (4.5% min) and for gradient delay effects (5.9 min). The gradient delay effects include the programmed gradient delay, the column void volume and the pre-column void. Therefore, Fig. 2 is a direct and simple comparison of the retention order of small peptides on PLRP-S with that on silica-based alkyl-bonded phases. No corrections are made for such effects as molecular weight[9] or changes in retention due to gradient rate, because for small peptides on relatively large pore materials, the effects of molecular weight are known to be far less dominant than hydrophobicity[9],

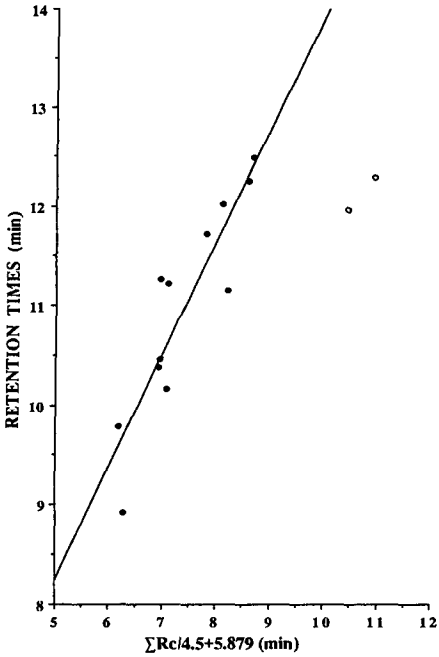

Fig. 2. Peptide retention on poly(styrene–divinyl benzene) *versus* alkyl-bonded phases. The retention (in minutes) predicted by the theory of Hodges and co-workers[9–11] is indicated by $\sum R_c/4.5 + 5.879$ and graphed on the abscissa, while the observed retention (in minutes) is graphed on the ordinate. Twelve peptides, including GFL, YY, YF, GLY, YGGFM, YV, YGGFL and YAGFM, YGGFLK, GGFM, FV and YL, appear to correlate well with the theory of Hodges and co-workers, and are indicated with filled circles (●). Two peptides which do not correlate well with the theory, RVYIHPF and DRVYIHPFHL, are indicated with hollow circles (○).

as are the effects of gradient rate[8]. Also, a mixture of GYG, PY, YV, YY, GLY, YF, GFL, YGGFM and benzoic acid was chromatographed at gradient rates of 2.25–9% acetonitrile/min and no inversions of retention were observed. The theory of Hodges and co-workers[7–9] assumes that the hydrophobicities of peptide side chains and termini contribute additively to retention time and that effects of alkyl chain length, column length, particle size and carbon loading can all be accounted for by the inclusion of a single standard. Fig. 2 shows that, to a first approximation, the same assumptions hold for PLRP-S and that retention can be predicted by additive retention coefficients.

Of the 21 peptides tested, seven (GYG, YG, PY, GY, YA, YGG and YE) are predicted by the theory of Hodges and co-workers not to be retained on column and these peptides do elute either isocratically or very early in the gradient, Fourteen (GFL, YY, YF. GLY, YGGFM, YV, YGGFL, YAGFM, YGGFLK, GGFM, FV, YL, RVYIHPF and DRVYIHPFHL) are predicted to be retained on column. Of these fourteen, all except the latter two appear to obey the linear behavior predicted in the theory of Hodges and co-workers. The peptides RVYIHPF and DRVYIHPFHL are slightly less strongly retained than predicted by theory. So, PLRP-S appears to exhibit the same general pattern of selectivity observed in alkyl bonded

phases, although it may be desirable to recalculate retention coefficients specifically for PS–DVB.

Another aspect of selectivity is the dependence of the capacity on the conditions of elution. Modern gradient theory[10–13] describes this dependence by means of graphs of the logarithm of the median solute capacity factor, log $\bar{k}$, on the median organic modifier mole fraction, $\bar{\varphi}$. The data required for such plots can be obtained by measurement of retention time on systematic variation of the gradient rate at constant flow-rate. The tryptic digest of horse cytochrome *c* was chromatographed at 1 ml/min on a gradient from aqueous–organic (95:5) (0–3 min) to aqueous–organic (60:40) mobile phase over a period of 40, 20, 10, 5 and 2,5 min. The data obtained from the plot of log $\bar{k}$ *vs.* $\bar{\varphi}$ are given in Table I.

TABLE I

DATA OBTAINED FROM THE PLOT OF log $\bar{k}$ *VS.* $\bar{\varphi}$

Cytochrome c peptide	*Retention time (min)*[a]	*Intercept*	*Slope*
1	16.3	0.34	−3.6
2	17.4	0.43	−3.8
3	19.9	0.53	−4.0
4	25.2	0.72	−5.3
5	25.9	0.76	−5.3
6	27.3	0.88	−4.8
7	34.8	1.3	−6.1
8	35.1	1.3	−5.8
9	37.8	2.2	−9.0
10[b]	38.9	1.6	−7.1
11	40.7	1.7	−7.2
12	42.3	1.8	−6.9

[a] Retention times (min) obtained from Fig. 1 (middle panel).
[b] Heme-containing peptide.

At gradient rates greater than 7% min, curvature was seen in the plots. The magnitudes of the slopes (and intercepts) are smaller than those generally seen in studies of peptide separations on bonded phase materials[12,13] and closer to the values seen for small organic molecules[14]. For YGGFM, values of −4.8 and 1.1 were obtained for the slope and intercept; these values are likewise smaller than those reported for separation on C_{18}-bonded phases[12,13]. Since the magnitude of the slope is believed to be related to the molecular weight[12,13], the implication would seem to be that peptides are less tightly and less cooperatively adsorbed to PS–DVB than to C_{18}-bonded phases.

Peak capacity

Plasminogen, comprised of 790 amino acids and bearing sites of glycosylation, is one of the most complex single-chain proteins. Fig. 3 shows the separation of plasminogen tryptic fragments as a function of gradient rate. There are noticeable changes in the fingerprint at different gradient rates, although careful examination reveals that common features are preserved.

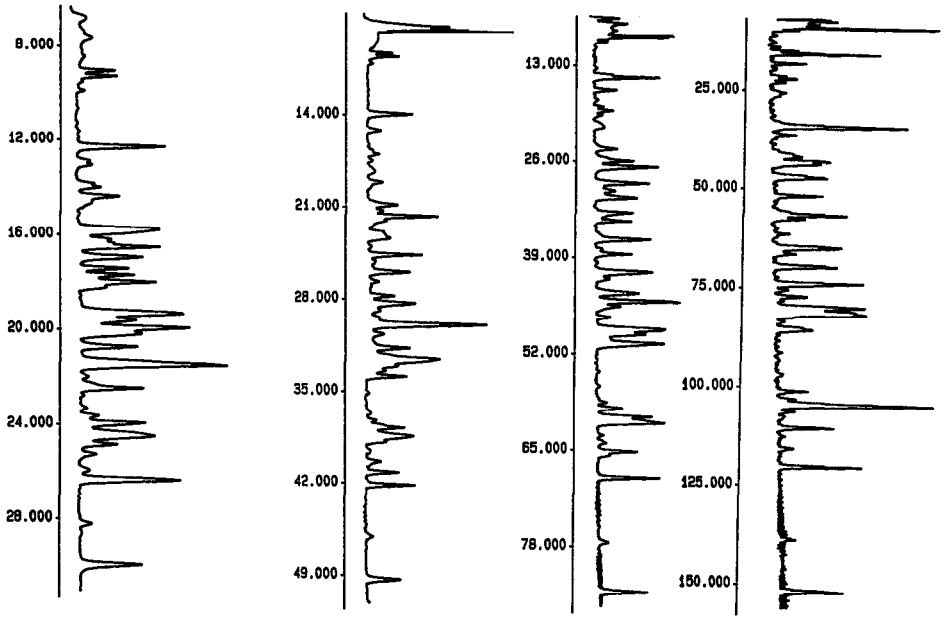

Fig. 3. Gradient rate dependence of tryptic fingerprints of human Glu-plasminogen. Reversed-phase separation of tryptic fragments of about 350 μg human plasminogen on a 25 × 0.46 cm I.D. 5-μm 100-Å PLRP-S column, using acetonitrile–water–0.1% trifluoroacetic acid–2-propanol as the mobile phase. Gradient conditions were aqueous-organic (95:5) (0–3 min) to aqueous–organic (90:10) (3–5 min), to aqueous–organic (50:50) over a variable period. From upper to lower panels, the gradient times were 4 h, 2 h, 1 h, and 30 min. Flow-rate was, in all cases, 1 ml/min. The ordinate is absorbance at 220 nm and the abscissa is retention time in minutes.

The peak capacity, which is a measure of the resolving power of a separation system, is usually defined as the maximum number of peaks which can be resolved to baseline over the gradient range. In fingerprinting, the peak capacity must exceed the number of expected proteolytic fragments to maximize the probability that all peaks will be resolved. The peak capacities of the gradient separations examined were esti-

TABLE II

PEAK CAPACITIES AT TYPICAL GRADIENT RATES

Tryptic digest	*Gradient rate (%/min)*	*Peak capacity*
Cytochrome *c*	14	31
	7	44
	3.5	55
	1.75	73
	0.88	87
Plasminogen	1.33	81
	0.67	112
	0.33	130
	0.17	137

mated according to the usual definition[2], but subtracting the gradient delay time (Table II). The peak capacities at typical gradient rates are about those observed for typical alkyl bonded silica phases[2,12,15]. In Fig. 4 is graphed the peak width as a function of gradient rate. A simple relationship, $W_{0.5} = 0.19(\Delta\psi/t_G)^{0.55}$, where $W_{0.5}$ is the peak width at half height and $\Delta\psi/t_G$ is the gradient rate, appears to hold over a regime of gradient rates spanning separations lasting 2.5 min to 4 h.

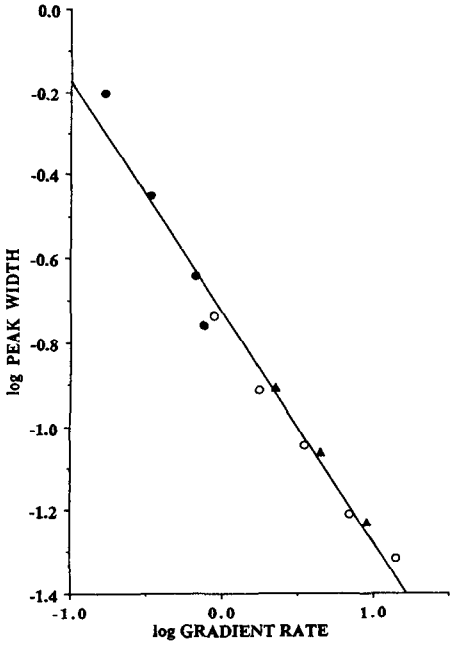

Fig. 4. Dependence of peak width on gradient rate. The peak widths at half height were measured for I: a mixture of PY, YV, YY, GLY, YF, GFL and YGGFM chromatographed at gradient rates of 2.25–9% acetonitrile per minute, II: those tryptic digest fragments of horse cytochrome *c* pictured in Fig. 1 which elute on the gradient and III: four well-resolved peaks of the tryptic digest of Glu-plasminogen pictured in Fig. 3 (retention items on the 4 h gradient 34.0, 56.6, 120.1 and 151.8 min). The logarithms of the average peak width are plotted against the logarithm of the gradient rate for the separation. I = △; II = ○; III = ●.

As is shown in Fig. 5, under conditions of high gradient rate, the performance of the PLRP-S column appears to improve slightly with an an increase in flow-rate. At 1.5 ml/min, the column pressure is at the maximum recommended by the manufacturer, *i.e.*, 3000 p.s.i., but still well below the column packing pressure. If one were to interpret this observation according to the Knox equation[16], it would seem to indicate that band broadening in fingerprint mapping on PLRP-S might not be dominated by mass transfer effects, but rather by diffusional broadening, presumably in the mobile phase. At present the effect is merely noted as an empirical observation and it is recommended that users investigate increased flow-rate as a means of improving resolution.

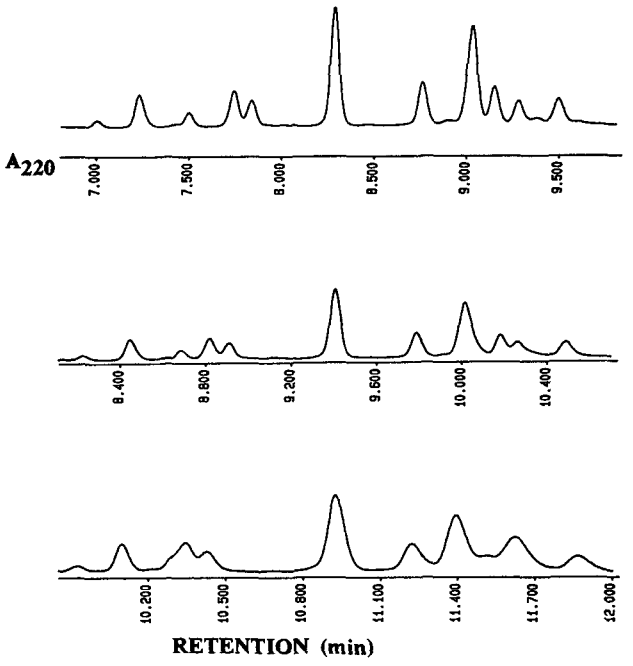

Fig. 5. Separation of tryptic fragments of horse cytochrome *c* as a function of flow-rate. Separation was performed on a 25 × 0.46 cm I.D. 5-μm 100-Å PLRP-S column, using acetonitrile–water–0.1% TFA–2-propanol as the mobile phase. Gradient conditions were aqueous-organic (95:5) (0–3 min) to aqueous–organic (60:40) over a variable period, with the gradient rate being 5% acetonitrile/min. Flow-rate was 1.5 (upper panel), 1.0 (middle panel) or 0.7 ml (lower panel). The ordinate is absorbance at 220 nm and the abscissa is retention time in minutes.

CONCLUSIONS

A number of factors may contribute to variability in reversed-phase fingerprint mapping. Among these factors are deamidation and peptide bond hydrolysis, both of which processes are time-dependent and accelerated at low pH and high temperature. Some of the recent approaches to rapid fingerprinting, which may require high temperatures and the addition of surfactants for optimal performance[3] may not be appropriate if fractions are to be collected and analyzed.

Pump performance, due to variation in flow-rate or gradient formation, is also an important factor in obtaining reliable fingerprints. The present manuscript has shown that small changes in gradient rate cause only small changes in fraction capacity and order of elution, but small changes in flow-rate can cause notable changes in the peptide fingerprint. The present study has shown that 100-Å, 5-μm PLRP-S exhibits selectivity equivalent to that of alkyl-bonded phases. The retention order is relatively insensitive to changes in gradient rate or flow-rate, making this phase suitable for rapid fingerprints of simple proteins. The peak capacity is about the same as that of other alkyl bonded phases. Although additional peak capacity is certainly desirable for complex fingerprints, such as plasminogen, PLRP-S is an excellent material for peptide mapping.

ACKNOWLEDGEMENTS

K. D. Nugent and F. P. Warner are thanked for review of this manuscript.

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