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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LVI*. DETERGENT-MEDIATED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF POLYPEPTIDES AND PROTEINS

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SUMMARY

The effects of different non-ionic polyoxyethylene alcohol surfactants on the retention behaviour of a series of polypeptides, chromatographed on octadecylsilica reversed-phase columns, are described. With aqueous–organic solvents at low pH as mobile phases these surface-active reagents produce significant decreases in retention of polypeptides on these microparticulate hydrocarbonaceous column packings at levels below 0.01% (w/v) without generally compromising selectivity. These trends are discussed in relation to the dependency between the logarithmic capacity factor of an ionised polypeptide and the surface tension of the mobile phase. The application of these non-ionic reagents in the separation and analysis of hydrophobic polypeptides under isocratic and gradient elution conditions is discussed.

INTRODUCTION

The purification of peptides or proteins from biological extracts usually requires the combination of several high-resolution liquid chromatographic procedures. Following the introduction over the past several years of suitable reversed-phase high-performance liquid chromatography (HPLC) procedures (for compendia of methods see refs. 1–3), rapid selective separations of a large variety of polypeptides and small proteins have now become possible. Selectivity in these separations is essentially based on differences in relative hydrophobic contact areas, established between the solutes and the stationary phase. Since the mobile phase composition in reversed-phase HPLC may be readily varied, numerous experimental options for controlling selectivity and recovery can be exploited. These options include pH modulation[4–6], the choice and concentration of different ionic buffers or modifiers[7–10] and manipulation[1,2,11] of the water content of the eluent under isocratic or gradient elution conditions.

The use of anionic and cationic surface-active reagents[1–4,12], such as sodium

* For Part LV see ref. 28.

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hexanesulphonate, sodium dodecylsulphate, tetrabutylammonium phosphate or dodecylammonium acetate has, in particular, considerably expanded the potential of reversed-phase HPLC in peptide and polypeptide fractionation. Usually, these reagents are employed at low concentrations, often in the range 0.1–15 m*M*, as mobile phase additives and exert their effects through composite pairing ion/dynamic liquid–liquid ion-exchange effects. Detailed investigations from this and other laboratories have demonstrated[4,8,13,14] that peptide retention with microparticulate, porous alkylsilicas may be varied independently of selectivity by changes in the mobile phase concentration of these hetaeric[15,16] reagents. The present investigation explores the use of non-ionic surface-active reagents, such as the aryl and alkyl (polyoxyethylene ether) alcohols in the reversed-phase HPLC of peptides and polypeptides. The results confirm that these surfactants are extracted by the non-polar stationary phase and exert their influence by decreasing the interfacial surface tension between the polar, water-rich mobile phase and the hydrocarbonaceous *n*-alkyl ligand.

MATERIALS AND METHODS

Apparatus

All chromatographic experiments were performed with a Waters Assoc. (Milford, MA, U.S.A.). liquid chromatographic system consisting of two M6000A pumps, a M660 solvent programmer, a U6K universal liquid chromatograph injector, a M450 variable-wavelength UV monitor, a M730 data module and a Model 3390A integrator Hewlett-Packard (Corvallis, OR, U.S.A.). Sample injections were made with SGE Model 50A syringes (SGE, Melbourne, Australia). Filtration of solvents was carried out using Pyrex filter holder (Millipore, Bedford, MA, U.S.A.) fitted with 0.4-μm membrane filters. The octadecylsilica used in this investigation was a Zorbax 6 μm octadecylsilica, maximally end-capped with hexamethyldisilazine, of average pore diameter 10 nm, surface area 177 m^2/g, and ligand density 2.2 μmol dimethyloctadecylsilyl groups/m^2, packed into 25 × 0.4 cm I.D. stainless-steel columns by means of the balanced density packing method. The pH measurements were carried out with a Radiometer PHM 64 meter equipped with a glass electrode.

Reagents

Water was quartz-distilled and deionised in a Milli-Q system (Millipore). Acetonitrile was HPLC grade, purchased from Waters Assoc. Potassium dihydrogen phosphate and orthophosphoric acid were AnalaR grade reagents from BDH (Poole, U.K.). The source of the polypeptides and proteins has been given previously[11]. Triton X-100 and Brij 35 were obtained from Sigma (St. Louis, MO, U.S.A.).

Methods

All chromatograms were carried out at room temperature (*ca.* 18°C). All polypeptides and proteins were dissolved in the eluent prior to injection. The sample loads for the dependency measurements were generally between 1 μg and 5 μg and were within the linear region of the adsorption isotherm. Bulk solvents were degassed separately, and the appropriate isocratic or gradient mobile phases were prepared as reported previously[11]. Following a change to a new mobile phase system, all columns

were equilibrated to the new conditions for at least 600–700 ml at a flow of 2 ml/min. The capacity factors, k', were calculated by conventional methods on the basis of $t_{sec}(NaNO_3)$ as t_o.

RESULTS AND DISCUSSION

Since their introduction[4,9,11,12] several years ago, the alkyl-sulphonates and -sulphates and the alkylammonium phosphates and acetates have greatly expanded our capabilities for the resolution of peptides and proteins by reversed-phase HPLC procedures. Recent investigations[2,9,13,14,17,18] on the mechanism of retention of peptides and related polar solutes on *n*-alkylsilicas, when such monopolar hydrophobic pairing reagents are used, have established that the extent of retention enhancement or attenuation can be closely correlated with the surface concentration and charge of the adsorbed pairing reagent. In related studies[1,12,19,20], we have demonstrated that other surface-active, non-ionic reagents, such as long-chain alkylalcohols and alkylnitriles can also be employed in low concentrations to modulate peptide and protein retention on alkylsilicas through hydrogen bonding and dipole–dipole interactions. For many years non-ionic detergents have been employed in conventional protein isolation procedures particularly as an aid to the solubilisation of hydrophobic membrane proteins. In view of the importance which the mobile phase bulk surface tension plays[11,21] as a key parameter in controlling the retention of polypeptides and proteins under regular reversed-phase HPLC elution conditions, we argued that low concentrations of non-ionic detergents added to aqueous–organic

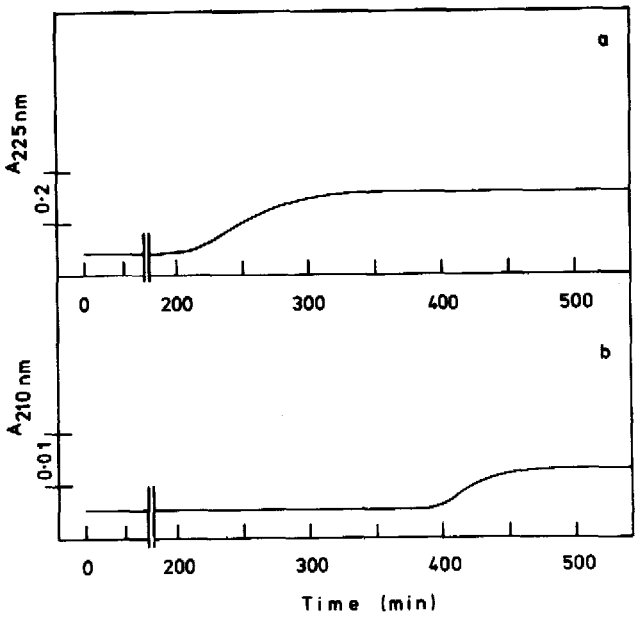

Fig. 1. Breakthrough patterns of eluents containing (a) 0.01% (w/v) Triton X-100 and (b) 0.01% (w/v) Brij 35, after equilibration of a reversed-phase column with acetonitrile–(30:70)–50 m*M* KH_2PO_4–15 m*M* H_3PO_4, pH 2.3, mobile phase; stationary phase, 6 μm octadecylsilica; flow-rate, 1.5 ml/min.

solvents as eluents would result in further decreases in retention without significant losses of selectivity. The present investigations confirmed these expectations.

Provided the distribution isotherm for a component is within a linear range, *i.e.* provided the adsorption isotherm obeys the Freundlich equation, then the breakthrough volume of that component in a bulk eluent should be equivalent —according to current concepts on chromatographic distribution processes— to the retention volume after injecting a small amount of that component. The breakthrough patterns for Triton X-100 and Brij 35 on the 6 μm octadecylsilica columns equilibrated with acetonitrile–water (30:70 v/v)–50 m*M* KH_2PO_4–15 m*M* H_3PO_4 are shown in Fig. 1. Based on an average monomer molecular weight of 650[22] for the *p-tert.*-octylphenol (polyoxyethylene) alcohol, Triton X-100, which exhibits a Poisson distribution of the various polyoxyethylene chain lengths centred at $n = 9$–10, the amount (37.1 mg) of Triton X-100 bound to the particular octadecylsilica column used, after equilibration with the above eluent containing 0.01% Triton X-100, is equivalent to *ca.* 17.8 μmol Triton X-100 per gram of octadecylsilica. Since the surface area of this particular bonded octadecylsilica is *ca.* 180 m^2/gm, the calculated surface coverage of Triton X-100 under the above mobile phase conditions is 0.10 μmol Triton X-100/m^2 or, alternatively, on an average there are *ca.* six Triton X-100 molecules per 100 nm^2 of stationary phase surface in this mobile phase condition. Based on the reported value[23] of 48–54 $Å^2$ for the surface area occupied by a Triton X-100 molecule at neat air–water interface, *ca.* 200 Triton X-100 molecules per 100 nm^2 of stationary phase surface would be anticipated as a limiting value of an adsorbed monolayer for a neat aqueous mobile phase, containing a concentration of Triton X-100 at or near the critical micelle concentation. In fact, neat aqueous conditions yield surface coverages of Triton X-100 *ca.* 30 times greater than those observed with the above binary aqueous–acetonitrile mobile phase where the volume fraction of the organic solvent was fixed at 0.3. Similarly, the amount (73 mg) of the lauryl (polyoxyethylene) alcohol, Brij 35, bound to the octadecylsilica column after equilibration with the same mobile phase but containing instead 0.01% Brij 35, is equivalent to *ca.* 10 μmol Brij 35 per gram of octadecylsilica, assuming an average monomer molecular weight of 1200 for Brij 35. The calculated surface coverage of Brij 35 under these isocratic conditions is thus 0.11 μmol Brij 35/m^2 or, on average, 6–7 Brij 35 molecules per 100 nm^2 of stationary phase surface.

The above data and related experiments suggest that the number of molecules, n_s, adsorbed at the interface per unit of surface area (in $Å^2$) progressively decreases from a limiting value, $n_{s,o}$, for a monolayer coverage in neat water as the volume fraction of the organic solvent in the mobile phase is increased. This change can be described by the Langmuir equation for the adsorption of the non-ionic detergent from a binary water–organic solvent combination onto a homogeneous surface, namely

$$\frac{1}{n_s} = \frac{1}{n_{s,o}} + \frac{1}{k\,\psi} \tag{1}$$

where

$$\frac{1}{\psi} = e^{-\frac{\Delta G}{RT}(A - A_o)A_o^{-1}} \tag{2}$$

and A is the area (Å^2) occupied by the adsorbed molecule at the interface at a particular organic solvent content in the mobile phase, A_o is the limiting value of A at monolayer coverage, K is a constant, ψ is the mole fraction of the non-ionic component in the bulk mobile phase and ΔG is the free energy of adsorption. When the bulk concentration of a non-ionic surfactant reaches the critical micelle concentation (CMC) the area per surfactant molecule adsorbed at the water–air or the water–hydrocarbon interface is anticipated to become constant with the value of n_s corresponding to this limiting area, A_o. At lower concentrations, the adsorption isotherm will approximately obey the Freundlich equation. Similar behaviour is also anticipated for ionic detergents, and has been previously documented[13] experimentally for alkylsulphonates. In contrast to anionic surfactants, such as the sodium salts of *n*-alkyl-sulphonates or -sulphates, non-ionic detergents derived from alkyl- and aryl-polyoxyethylenne alcohols of the Brij and the Triton series often have CMC values *ca.* 10–100 times smaller. For example, the CMC value and aggregation number of sodium dodecylsulphate[24] in neat water are 8.2 mM and 62, respectively, whilst the corresponding values for Triton X-100[25] in neat water are 240 μM and 140. When the monomer concentration exceeds the CMC value of a non-ionic surfactant at a particular temperature and eluent composition, thermodynamically stable aggregates will spontaneously form by processes mediated essentially through the driving force of weak dispersive hydrophobic interactions. Although the chosen concentrations of Triton X-100 and Brij 35 over which the k'-values of the polypeptides greatly decreased were in the above experiments well below the CMC values in neat water for these two detergents, clearly further potential exists[26,27] for reversed-phase chromatographic separation of polypeptides and proteins under micellar conditions to affect selective elution. Depending on the organic solvent content, ionic strength and temperature, final concentrations of these surfactants in excess of their CMC in neat water, *e.g.* greater than 240 μM Triton X-100, would be required for micelles to form under binary water–organic solvent chromatographic conditions similar to those employed above.

Three important features germane to the above discussion were immediately noticable from these experiments. Firstly, the breakthrough patterns for these two non-ionic detergents were found to be dependent on the water content of the mobile phase in accord with chromatographic distribution theory. When a very low organic solvent content was chosen for the mobile phase, following the initial column equilibration the breakthrough volumes for the detergents became very large. As a consequence, considerable care had to be taken to ensure that the columns were properly equilibrated to each new mobile phase condition. Secondly, if UV detection at low wavelengths, *i.e.* below 225 nm is to be employed, the alkyl polyoxyethylene alcohols, such as the lauryl derivative, Brij 35, are the preferred surfactants over the corresponding aryl derivatives. Thirdly, as the hydrophobicity of the non-ionic detergent increased, the breakthrough volume also increased. As a consequence, considerable potential exists for varying retentions through the use of non-ionic detergents of different polyoxyethylene chain length in a manner analogous to the use of *n*-alkyl-sulphonate, -sulphate or -ammonium salts of different alkyl chain length[2,4,9,12,14].

Figs. 2–4 show the plots of the retention characteristics of several phenylalanine oligomers, hormonal peptides and polypeptides up to 14,000 daltons, separated isocratically on octadecylsilica columns with a fixed water–acetonitrile composition

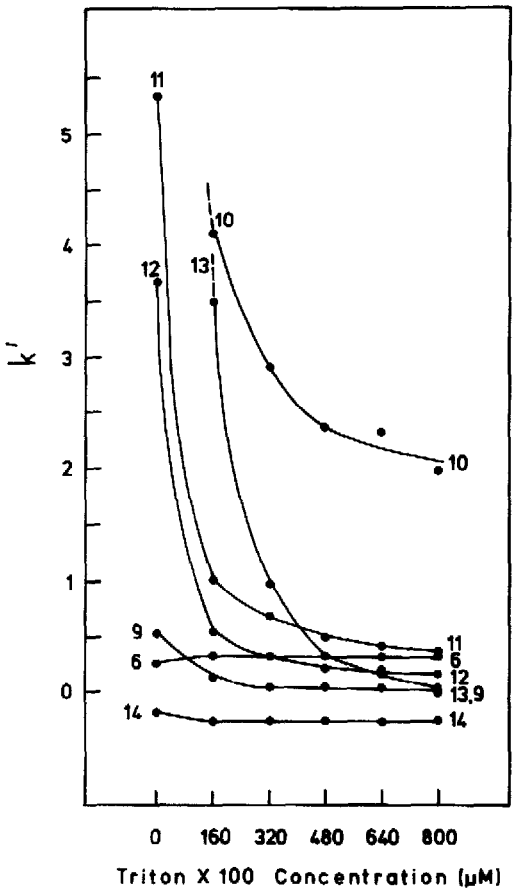

Fig. 2. Dependence of the capacity factors of protonated polypeptides on the concentration of the non-ionic detergent Triton X-100 in the mobile phase. Chromatotographic conditions: column, octadecylsilica; flow-rate, 1.0 ml/min; temperature, 18°C; mobile phase, acetonitrile–water (30:70)–50 m*M* KH_2PO_4–15 m*M* H_3PO_4, pH 2.3, containing various concentrations of the detergent. See Table I for polypeptide key.

in the presence of varying amounts of the two non-ionic detergents. With both Triton X-100 and Brij 35 dramatic decreases in retention were evident when the concentration of the detergent in the mobile phase was increased over the range 0–0.01% (w/v), *i.e.* up to 164 μ*M* Triton X-100 and 96 μ*M* Brij 35, respectively. For the small peptides, such as the phenylalanine oligomers, and for the larger polypeptides reversed phase selectivity was maintained, with the peptides being eluted in order of increasing hydrophobicity. The most noticeable exception among the larger polypeptides was cytochrome *c*, which was eluted much more rapidly than would be anticipated solely on the basis of decreased hydrophobic interactions with the stationary phase. Peptidic solutes, such as tri.tyrosine, which were weakly retained on the octadecylsilica column in the absence of a non-ionic detergent, exhibited little change in retention over the range of detergent concentrations examined. Nevertheless, most solutes were slightly retained at the highest Triton X-100 or Brij 35 concentration employed.

The above results demonstrate that non-ionic detergents in low concentrations

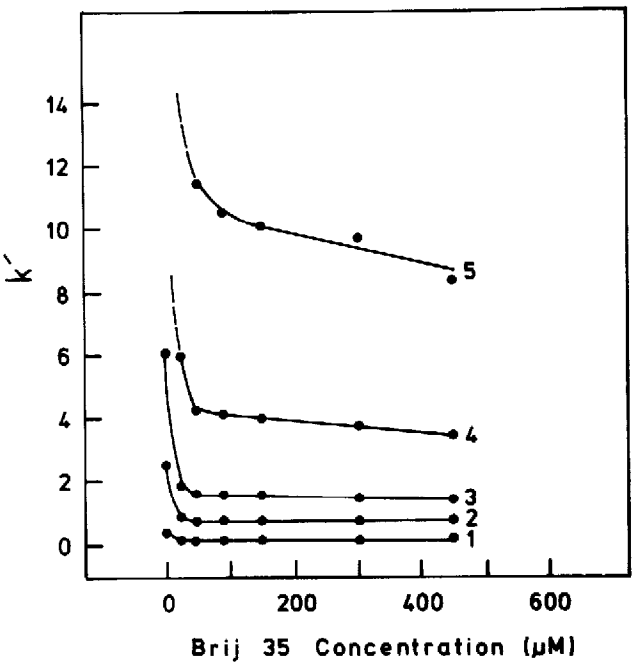

Fig. 3. Plots of the dependence of the capacity factors of protonated phenylalanine oligomers on the concentration of Brij 35 in the mobile phase. Chromatographic conditions are the same as those given in the legend to Fig. 2. See Table I for peptide key.

exhibit important properties useful in reversed-phase HPLC of peptides and proteins. These reagents have the ability to act as solubilising agents and, at the concentrations employed here, do not generally impair biological function of proteins or interfere in most methods of structural analysis, *e.g.* amino acid composition determination, sodium dodecylsulphate–polyacrylamide gel electrophoresis, etc. In particular, these surface-active reagents reduce both the surface tension of the bulk mobile phase as well as the interfacial surface tension between the water-rich eluent and the hydrocarbonaceous ligand. Previous studies[11,21] have demonstrated that retention of polyelectrolytes in reversed-phase HPLC is directly related to the interfacial and bulk surface tensions. Experimental results have confirmed that the relationship between the logarithmic capacity factor, log k', of small polypeptides and the mobile phase surface tension, γ_m, can be approximated by a linear dependency over the range of organic solvent compositions commonly used in reversed phase HPLC procedures. As a consequence, non-ionic reagents of low polarity which augment surface tension decreases may in general be of considerable benefit in the separation and recovery of hydrophobic membrane proteins, large CNBr-fragments or large tryptic peptides which cannot be satisfactorily eluted from alkylsilicas with conventional low pH water–organic solvent combinations. For example, at concentrations of 0.001% (w/v) and 0.01% (w/v) Triton X-100 reduces the surface tension of distilled water from 72 dyn/cm to 46 dyn/cm and 30 dyn/cm, respectively. To achieve a similar surface tension decrease with a water–acetonitrile combination would require *ca.* 13% and 50% acetonitrile, respectively. In view of the pronounced sensitivity[3,11] of protein reten-

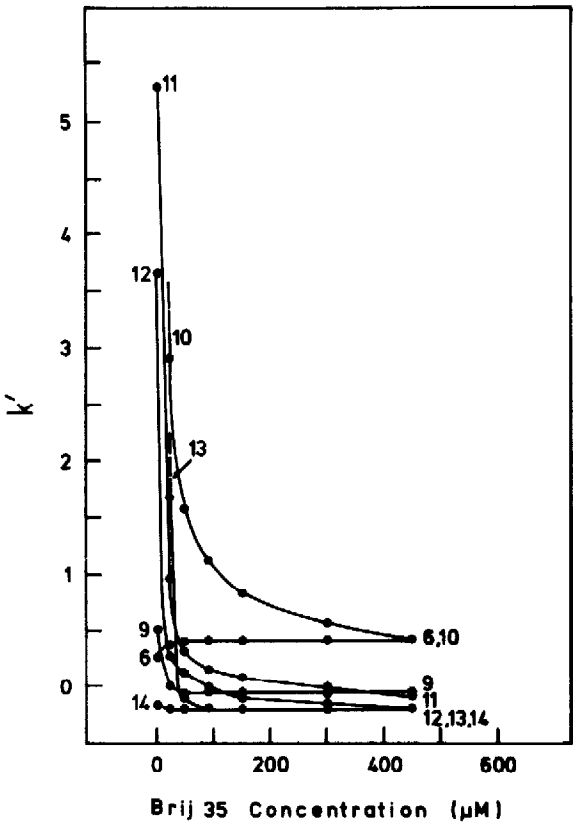

Fig. 4. Plots of the dependence of the capacity factors of protonated polypeptides on the concentration of Brij 35 in the mobile phase. Chromatographic conditions are the same as those given in the legend to Fig. 2. See Table I for the polypeptide key.

tion and recovery to the organic solvent content in reversed-phase HPLC, unacceptable chromatographic performance often can occur at inappropriate solvent compositions. Typical of this behaviour were results obtained during the present investigation for several of the polypeptides which could not be eluted in the absence of a detergent with an acetonitrile–water (30:70)–50 m*M* KH_2PO_4–15 m*M* H_3PO_4 mobile phase but were readily recovered with good peak shapes when less than 0.01% (w/v) detergent was added to the same mobile phase.

Finally, the results of these investigations have several practical implications for gradient elution separations of polypeptides under reversed-phase conditions. Since the amount of a non-ionic detergent bound by the column will in general be a function of the organic solvent mole fraction in the mobile phase, gradient elution from a water-rich to a water-lean mobile phase will result in considerable desorption of the bound detergent with concomitant UV absorption changes of the baseline as well as non-equilibrium interactions between the ionised peptidic solutes and the dynamically modified stationary phase. The former effect does not represent a significant limitation when detection of a particular polypeptide or protein component is based on a biological assay or, alternatively, when fluorescence detection with

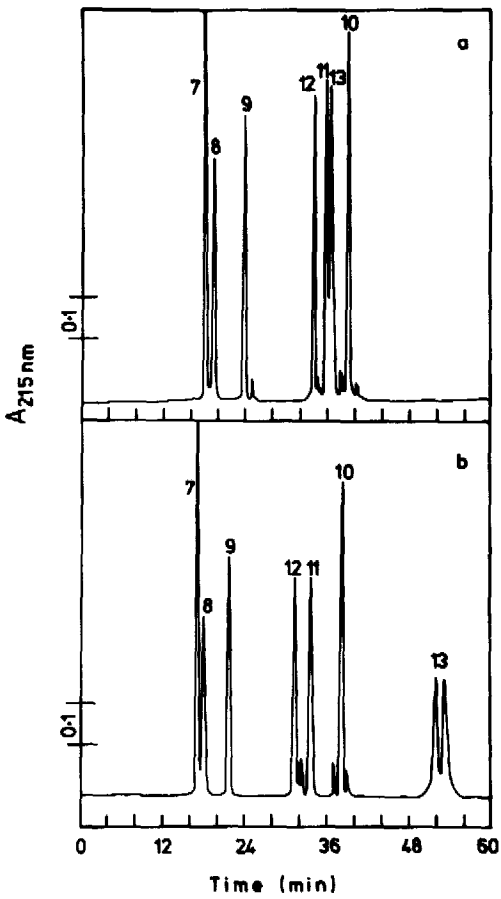

Fig. 5. Gradient elution separation of several polypeptides with an octadecylsilica column in (a) the absence or (b) the presence of 0.01% (w/v) Brij 35. Chromatographic conditions: column, octadecylsilica (10 nm porosity); flow-rate, 1.0 ml/min; initial mobile phase conditions, acetonitrile–water (15:85 v/v)–50 m*M* sodium dihydrogen phsophate–15 m*M* orthophosphoric acid; final mobile phase conditions, acetonitrile–water (50:50)–50 m*M* sodium dihydrogen phosphate–15 m*M* orthophosphoric acid. The detergent was added at 0.01% (w/v) to both the initial and final conditions in (b). Linear gradient from initial to final conditions over 60 min. See Table I for key to polypeptides.

post-column derivatisation with a fluorogenic reagent, such as fluorescamine, is employed. However, the second effect can result in variable retention behaviour and resolution for polypeptides separated under these conditions. It is thus essential that care is taken to ensure that the columns are thoroughly re-equilibrated at the completion of each gradient experiment and that optimised gradient steepness conditions with regard to flow and gradient rate are employed. However, as is evident from Fig. 5, gradient elution of polypeptides can be achieved under detergent-mediated reversed-phase HPLC conditions even with universal UV detection at 215 nm. The origin of the double peak for cytochrome *c* as well as the selectivity reversal (compared to glucagon) in the presence of Brij 35 is at this stage unclear. However, it is worth recalling that cytochrome *c* and other basic, hydrophobic proteins can exihi-

TABLE I

PEPTIDES AND POLYPEPTIDES USED IN THE PRESENT STUDY ON DETERGENT CONCENTRATION DEPENDENCY

No.	*Peptide/polypeptide**	*No.*	*Peptide/polypeptide*
1	F	8	Angiotensin II
2	FF	9	Angiotensin I
3	FFF	10	Porcine glucagon
4	FFFF	11	Bovine insulin B chain
5	FFFFF	12	Bovine insulin
6	YYY	13	Cytochrome *c*
7	Angiotensin III	14	Hen egg lysozyme

* The one-letter code for the amino acids is used as given by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, NBRF, Silver Spring, MD, U.S.A., 1972.

bit[3,6] unusual elution behaviour in reversed-phase HPLC systems, particularly in circumstances where slow secondary chromatographic equilibria apply. In this context, the similar bandwidths obtained for polypeptides eluted under these gradient reversed-phase conditions in the presence and absence of non-ionic detergents would suggest that adequate control over kinetic phenomena can be achieved with these elution systems. Detailed examination of efficiency dependencies of polypeptides chromatographed on alkylsilicas and eluted with mobile phases containing non-ionic detergents will be reported elsewhere.

In summary, these investigations have confirmed that low concentrations of non-ionic surfactants significantly influence the retention of polypeptides on *n*-alkylsilicas. The most noticeable feature of the use of these reagents in the range 5–15 μmol/l is their ability to decrease the retention of a particular polypeptidic component. As a consequence, polypeptides are eluted at much lower organic solvent content with low pH binary aqueous–organic solvent mobile phases. Systematic use of these non-ionic modifiers should thus considerably expand the capabilities of reversed-phase HPLC for the resolution of complex mixtures of the more hydrophobic polypeptides or proteins during micro-preparative isolation and associated structural studies.

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REFERENCES

1 M. T. W. Hearn, *Advan. Chromatogr.*, 20 (1982) 1.
2 M. T. W. Hearn, in Cs. Horváth (Editor), *HPLC: Advances and Perspectives*, Academic Press, Vol. 3, Academic Press, New York, 1983, pp. 87–155.
3 M. T. W. Hearn, *Methods Enzymol.*, 104 (1983) 190.
4 M. T. W. Hearn, B. Grego and W. S. Hancock, *J. Chromatogr.*, 185 (1979) 429.
5 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
6 K. A. Cohen, K. Schellenberg, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, in press.

7 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
8 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liquid Chromatogr.*, 3 (1980) 1353.
9 M. T. W. Hearn, S. J. Su and B. Grego, J. Liquid Chromatogr., 4 (1981) 1547.
10 J. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
11 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
12 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *Science*, 200 (1978) 1168.
13 R. S. Deelder and J. H. M. Van den Berg, *J. Chromatogr.*, 218 (1981) 327.
14 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 203 (1981) 349.
15 Cs. Horváth and W. Melander, *J. Chromatogr. Sci.*, 15 (1977) 393.
16 Cs. Horváth, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
17 J. H. Knox and R. A. Hartwick, *J. Chromatogr.*, 204 (1981) 3.
18 R. S. Deelder, H. A. J. Linssen, A. P. Konijnendijk and J. L. M. van de Venne, *J. Chromatogr.*, 185 (1979) 241.
19 M. T. W. Hearn and W. S. Hancock, in G. L. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography*, Marcel Dekker, New York, 1979, pp. 243–271.
20 M. T. W. Hearn, submited for publication.
21 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 266 (1983) 75.
22 A. Helenius and K. Simons, *Biochim. Biophys. Acta*, 415 (1975) 29.
23 Rohm and Hass Technical Bulletin EXSP-06 (1967) and EXS 63 b (1969).
24 P. Mukerjee and K. J. Mysels, *Critical Micelle Concentrations in Aqueous Surfactant Systems*, National Bureau of Standards, NSRDS-NBS 36, Washington, 1971.
25 L. M. Kushner and W. D. Hubbard, *J. Phys. Chem.*, 58 (1954) 1163.
26 M. T. W. Hearn, in preparation.
27 R. A. Barford and B. J. Sliwinski, personal communication.
28 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.