Journal of Chromatography, 361 (1986) 161–167
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 618

REVERSED-PHASE ION-PAIR CHROMATOGRAPHY OF BASIC, HYDROPHOBIC PEPTIDES

SUBSTANCE P AND FRAGMENTS

BENGT FRANSSON
Institute of Biochemistry, University of Uppsala, Biomedical Centre, Box 576, S-751 23 Uppsala (Sweden)
(Received February 10th, 1986)

SUMMARY

The effect of alkane sulphonates, varying in size from ethane to *n*-pentane, on the retention of substance P to alkylsilane-bonded (C_6, C_8 and C_{18}) microsilica columns with ethanol as organic modifier has been studied. Applying the same counter-ion in constant concentration, for this hydrophobic peptide the three columns gave essentially the same degree of retention. The sulphonate of *n*-pentane and *n*-butane and, to some extent, *n*-propane could be exploited for regulation of the retention, however, ethane sulphonate proved inefficient for this purpose.

The peak symmetry varied among the supports studied. Peaks with good symmetry were obtained only when fully capped reversed-phase supports were used. On the other hand, with partly capped supports the peak symmetry could be greatly improved by the addition of amines or quaternary ammonium compounds to the mobile phase.

A high separation efficiency ($h < 10$) was obtained with the fully capped alkyl silane-bonded supports.

INTRODUCTION

High-performance liquid chromatography (HPLC) is nowadays a well-established technique for characterization, quantitation and purification of both natural and synthetic peptides[1]. It is often superior to other methods with respect to speed and efficiency, as well as to convenience. Since, by definition, peptides are charged substances at most pH values, the presence of various counter-ions generally influences their chromatographic behaviour. The addition of proper counter-ions to the mobile phases has been used extensively to regulate the retention of peptides in HPLC[1].

We recently applied the ion-pair technique to the separation of basic, hydrophilic peptides by reversed-phase HPLC[2]. Although most of the compounds used in that process were extremely hydrophilic, no difficulties were encountered in making the corresponding cationic ion-pairs containing sulphonic acids hydrophobic enough

0021-9673/86/$03.50

to interact with the stationary phase and thus accomplish solute retention. The aim of the present work was to study the behaviour of a basic, hydrophobic peptide under similar conditions, and for this purpose substance P[3,4] was chosen.

MATERIALS AND METHODS

Isocratic mobile phases were used, consisting of binary aqueous solvents with ethanol and acetonitrile as organic modifiers. The buffers were prepared from orthophosphoric acid and sodium dihydrogenorthophosphate to an ionic strength of 0.1 *M*. *n*-Alkanesulphonates (C_2–C_5) were used as counter-ions in the concentration range of 0.01–0.05 *M*. 1-Pentane- and 1-butanesulphonic acids were obtained as sodium salts from Eastman-Kodak (Rochester, NY, U.S.A.). 1-Propanesulphonic acid as sodium salt and ethanesulphonic acid were purchased from Fluka (Buchs, Switzerland).

Substance P and its fragments, except the nonapeptide, were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). The nonapeptide was from Chemical Dynamics (South Plainfield, NJ, U.S.A.). All chemicals and solvents were of analytical or reagent grade when not otherwise stated.

The liquid chromatographic system consisted of a Model 6000 A solvent delivery device and a Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The flow cells had a volume of 8 μl and the detector head was carefully isolated in order to reduce any influence on the baseline stability at high sensitivity due too variations in the ambient temperature. The detection wavelength was 210 nm at high sensitivity. A Model 7125 sample loading injector was obtained from Rheodyne (Cotati, CA, U.S.A.).

The separation columns (150 × 4.6 mm I.D.) were packed with Spherisorb C_6, C_8, C_{18} (ODS-1 and ODS-2) and CN (cyanopropyl) reversed-phase supports (5 μm) obtained from Phase Separations (Queensferry, Clwyd, U.K.). According to the information from the manufacturer, ODS-1 is partially capped and ODS-2 is fully capped. The separation columns were always preceded by a short guard column (30 × 3.0 mm I.D.), dry packed with a pellicular support, μBondapak C_{18} (Corasil 37–50 μm, Waters), and thermostated at 30.0 ± 0.1°C using a home-made block heater. The retention studies and the separations were performed at a nominal flow-rate of 1.0 ml/min.

The retention studies of substance P on different columns were carried out with the same mobile phase composition by varying the nature and concentration of the counter-ion present in the mobile phase. Phosphate buffer (pH 3.0) was used as t_0 marker from baseline disturbances. No retention of the peptides was observed on the guard column.

RESULTS AND DISCUSSION

Reversed-phase ion-pair chromatography has recently been reviewed in a wider context[5]. A book on ion-pair chromatography with one chapter covering amino acids, peptides and proteins has recently been published[6]. Some selected earlier reports in the literature dealing more specifically with peptide separation based on the same principles have also been discussed and summarized[2]. Thus, in the studies of somatostatin, it was concluded that the capacity ratio was at least partly regulated by the counter-ion. Other retention mechanisms could, however, not be excluded[7].

Substance P is a strongly basic endogenous 11-peptide with three positive charges, and thus has the potential of carrying three anionic counter-ions. Spindel *et al.* previously applied an ion-pair approach to the analysis of substance P using *n*-pentanesulphonate as counter-ion on a C_{18} column[8].

Retention of substance P on solid supports with different alkyl ligands

In a first set of experiments, substance P was chromatographed on C_6, C_8 and C_{18} microsilica columns, all fully capped, using the same mobile phases, containing a series of *n*-alkanesulphonates as ion-pairing agents. The corresponding capacity factors are presented in Table I.

The nature of the counter-ion was varied from ethane to *n*-pentanesulphonate. Using the same counter-ion for the hydrophobic peptide, substance P, there were no significant differences between C_6, C_8 and C_{18} below a capacity factor (k') of 15. When the more hydrophobic anions were used, the capacity factor increased slightly in the order $C_6 < C_8 < C_{18}$ and was most pronounced at higher k' values. The retention could be regulated by the longer *n*-alkanesulphonates and, to a minor extent, by *n*-propanesulphonate, but ethanesulphonate was too hydropilic and gave retention values similar to those of phosphate alone. A high separation efficiency, $h < 10$, was obtained and it slightly increased in the order of $C_6 < C_8 < C_{18}$ at similar k' ratios when the same counter-ion was used.

The use of a partly coated solid support (ODS-1, 0.3 mmol/g) resulted in asymmetrical peaks for substance P with severe tailing effects, probably due to adsorption to the residual silanol groups. The symmetry of the substance P peaks could, however, be improved by the addition of tertiary amines[9] or small quaternary ammonium compounds[10] to the mobile phases. With these additives used in excess in the acidic pH range, most of the residual silanol groups were obviously blocked and thus pre-

TABLE I

k' VALUES OF SUBSTANCE P ON THREE DIFFERENT ALKYLSILANE-BONDED MICROSILICA SUPPORTS USING ALKANE SULPHONATES FOR ION PAIRING

Column: 150 × 4.6 mm I.D. Support: Spherisorb C_6, C_8 and C_{18} ODS-2, 5 μm. Mobile phase: 0.1 *M* phosphate buffer (pH 3.0)–ethanol (72:28). Counter-ions: alkanesulphonates, 0.01 *M* and 0.05 *M*, respectively. Flow-rate: 1.0 ml/min. Temperature: 30°C.

Counter-ion	*Capacity factors (k')*			
	C_xM	C_6	C_8	C_{18}
Ethanesulphonate	0.01	5.7	7.1	6.5
	0.05	6.6	6.9	7.5
n-Propanesulphonate	0.01	6.7	7.8	7.6
	0.05	9.0	10.0	11.7
n-Butanesulphonate	0.01	11.6	11.3	11.4
	0.05	22.2	25.0	30.5
n-Pentanesulphonate	0.01	24.0	27.9	29.9
Without counter-ion	–	5.9	7.3	5.6

vented adsorption, which also decreased the retention. The importance of an acidic pH to prevent ionization of the peptide carboxyl and residual silanol groups has been illustrated earlier[11].

Application of nitrile-bonded silica support (cyanopropyl, 0.6 mmol/g) was also attempted, but invariably resulted in very low k' values compared with the alkylsilane-bonded microsilica support. Moreover, with substance P as solute, asymmetrical peaks were obtained with pronounced tailing tendencies. The chromatographic conditions used were the same as in the previous cases with alkylsilane-bonded supports.

Retention of substance P on fully endcapped solid supports

With fully capped support (ODS-2, 0.5 mmol/g), the peak symmetry was further improved and no additives were required. The influence of capping on the recovery of proteins has previously been pointed out[12]. Fully capped columns appear to be a prerequisite in chromatography of more hydrophobic peptides, such as substance P, in order to get symmetrical peaks and high recoveries. This is contradictory to our experience with basic hydrophilic peptides, which could be run without disturbing tailing effects also on partly coated supports (ODS-1)[11].

Separation of substance P fragments with ethanol as organic modifier

The optimal chromatographic conditions found for substance P itself were finally applied to the separation of some substance P fragments, the structures of which are given in Table II.

A typical separation of substance P fragments is illustrated in Fig. 1, with *n*-butanesulphonate as counter-ion.

The corresponding retention values, selectivity and efficiency data are presented in Table III. Using *n*-butanesulphonate, all the components were well resolved and eluted in order of increasing number of amino acid residues, with the free acid coming last. It should be pointed out in this context that, in all experiments described in this paper, substance P eluted before its acid (SP-acid). Since at pH 3.0 the carboxyl group is partly dissociated, this can be taken as an indication of ion-pairing effects. As expected, the order of elution was drastically changed. Table III also contains results without an alkane sulphonate present in the mobile phase. In the phosphate system, the order of retention was in principle reversed, since the counter-ion, dihydrogenphosphate, is hydrophilic[13].

The obtained efficiencies were quite satisfactory ($h < 10$) with *n*-butanesulphonate present in the mobile phase, and were improved 2–3 times in comparison with the results obtained in the absence of sulphonates.

TABLE II

PEPTIDE STRUCTURES

SP = Substance P.

SP-acid	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
SP_{2-11}	Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
SP_{3-11}	Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
SP_{4-11}	Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

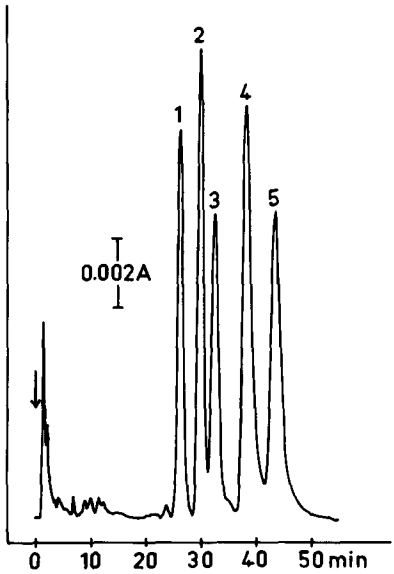

Fig. 1. Separation of substance P and fragments by reversed-phase ion-pair chromatography. Column, 150 × 4.6 mm I.D. Support, Spherisorb C_{18} ODS-2 (5 μm). Mobile phase, 0.1 *M* phosphate buffer (pH 3.0)–ethanol (72:28). Detection wavelength, 210 nm. Flow-rate, 1.0 ml/min. Temperature, 30°C. Counter-ion, 0.03 *M* *n*-butanesulphonate. Peaks: 1 = SP_{4-11}; 2 = SP_{3-11}; 3 = SP_{2-11}; 4 = substance P; 5 = SP-acid.

Separation of substance P fragments with acetonitrile as organic modifier

The separations of the substance P (SP) fragments were also studied using acetonitrile as organic modifier. The retention values, efficiency and selectivity data obtained are presented in Table IV. Using *n*-butanesulphonate, all the components were not fully resolved and eluted in the order SP_{3-11}, SP_{4-11}, SP, SP-acid and, lastly, the decapeptide SP_{2-11}. The peptides having a terminal basic amino acid residue were eluted earlier than the corresponding shorter peptide, in agreement with Rekker's

TABLE III

SEPARATION OF SUBSTANCE P AND FOUR CLOSELY RELATED DERIVATIVES BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY WITH AND WITHOUT *n*-BUTANESULPHONIC ACID PRESENT IN THE MOBILE PHASE USING ETHANOL AS ORGANIC MODIFIER

SP = Substance P; α = selectivity ratio. Chromatographic conditions as in Fig. 1, when not otherwise stated.

Peptide	*With sulphonate*			*Without sulphonate*		
	H	*k'*	α	*H*	*k'*	α
SP	0.05	22.0	1.47	0.12	6.9	1.00
SP_{2-11}	0.04	18.7	1.25	0.09	9.3	1.35
SP_{3-11}	0.04	17.7	1.19	0.07	7.9	1.15
SP_{4-11}	0.04	14.9	1.00	0.07	10.2	1.49
SP-acid	0.05	25.1	1.68	0.10	8.7	1.26

TABLE IV

SEPARATION OF SUBSTANCE P AND FOUR CLOSELY RELATED DERIVATIVES BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY WITH AND WITHOUT *n*-BUTANE SULPHONIC ACID PRESENT IN THE MOBILE PHASE USING ACETONITRILE AS ORGANIC MODIFIER

SP = Substance P; α = selectivity ratio. Column: 150 × 4.6 mm I.D. Support: Spherisorb C_{18} ODS-2, 5 μm. Mobile phase: 0.1 *M* phosphate buffer (pH 3.0)–acetonitrile (75.5:24.5). Flow-rate: 1.0 ml/min. Counter-ion: 0.03 *M* *n*-butanesulphonate. Temperature: 30.0 ± 0.1°C.

Peptide	*With sulphonate*			*Without sulphonate*		
	H	*k'*	α	*H*	*k'*	α
SP	0.04	22.7	1.29	0.09	4.5	1.00
SP_{2-11}	0.04	25.7	1.46	0.05	6.9	1.54
SP_{3-11}	0.03	17.6	1.00	0.06	5.0	1.11
SP_{4-11}	0.03	19.7	1.12	0.04	7.5	1.68
SP-acid	0.05	24.7	1.41	0.08	5.0	1.11

fragmental constants in liquid–liquid partition systems[12]. The retention values with *n*-butanesulphonate present were increased compared with those in phosphate buffer alone. The order of elution in phosphate was changed to SP, SP-acid and SP_{3-11}, SP_{2-11} and then the octapeptide, SP_{4-11}, eluting last with respect to the relative selectivity between the pairs SP_{2-11}/SP and SP_{4-11}/SP_{3-11}, with as difference of one charge unit; moreover, a proline residue following the basic amino acid in both cases, decreased from 1.5 to 1.1 on the addition of *n*-butanesulphonate to the mobile phase. This means that the hydrophobic counter-ion, *n*-butane sulphonate, like the hydrophilic counter-ion, dihydrogenphosphate, decreased the peptide retention. The efficiency data are quite satisfactory with *n*-butanesulphonate present, *i.e.* 2–3 times improved compared with the phosphate system alone.

CONCLUSIONS

In our study we have limited the experimental work to compare different reversed-phase supports, counter-ions, modifiers and the separation of substance P fragments and left the study of retention mechanisms for a later work.

For the hydrophobic peptide, substance P, the optimal chromatographic behaviour was only obtained on fully capped supports.

It was recently proposed that C_6, C_8 and C_{18} present similar interactive surfaces to peptides because the alkyl chains are "folded back" upon the silica support in water-rich mobile phases[15]. This explanation appears to be valid also for the experiments presented in Table I.

The retention of substance P could be regulated with the sulphonates of *n*-pentane and *n*-butane and, to some extent, with *n*-propane as shown for the capacity ratios obtained in Table I.

Due to the amino acid composition of the peptides, ranging from weakly to strongly basic fragments and having positive charges from one to three units in the acidic range, which implies the potential of carrying the corresponding number of

counter-ions, a discussion of retention mechanisms becomes rather complex. According to the basic principle of ion-pair chromatography on reversed-phase supports using hydrophilic modifiers, as in our case, we refer to the ion-pair adsorption model[16,17].

As organic modifiers with alkanesulphonates present, both ethanol and acetonitrile improved the efficiency compared with the phosphate system. Acetonitrile was superior in this respect, whereas the selectivity was better with ethanol. The addition of a hydrophobic counter-ion increased the retention of the hydrophobic peptides used. The use of either ethanol or acetonitrile is almost comparable due to the fact that the resolution is dependent on both the selectivity and efficiency obtained at similar capacity ratios well known from the basic equation for resolution[18].

The comparison between ethanol and acetonitrile as organic modifiers has been performed at similar k' values, which show that less acetonitrile had to be used compared with ethanol. This is in agreement with established data for small molecules.

In general, the use of hydrophobic ion-pairing agents is of greater importance for basic hydrophilic peptides than for the comparatively large undecapeptide, substance P. On the other hand, the symmetry, efficiency and selectivity can be improved by this technique, thus enabling a satisfactory resolution of closely related hydrophobic peptides.

ACKNOWLEDGEMENTS

Professor Douglas Westerlund, Department of Analytical Pharmaceutical Chemistry, and Dr. Ulf Ragnarsson, Institute of Biochemistry, are gratefully acknowledged for valuable criticism of the manuscript.

REFERENCES

1 W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vols. 1 and 2, CRC Press, Boca Raton, 1984.
2 U. Ragnarsson, B. Fransson and Ö. Zetterqvist, in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. 2, CRC Press, Boca Raton, 1984, p. 75.
3 U. S. von Euler and J. H. Gaddum, *J. Physiol.*, 72 (1931) 74.
4 M. M. Chang, S. E. Leeman and H. D. Niall, *Nature (London), New Biol.*, 232 (1971) 87.
5 R. E. Majors, H. G. Barth and C. H. Lochmüller, *Anal. Chem.*, 56 (1984) 321 R.
6 M. T. W. Hearn (Editor), *Ion-pair Chromatography*, Marcel Dekker, New York, 1985.
7 M. Abrahamsson and K. Gröningsson, *J. Liq. Chromatogr.*, 3 (1980) 495.
8 E. Spindel, D. Pettibone, L. Fisher, J. Fernstrom and R. Wurtman, *J. Chromatogr.*, 222 (1981) 381.
9 K.-G. Wahlund and A. Sokolowski, *J. Chromatogr.*, 151 (1978) 299.
10 M. E. F. Biemond, W. A. Sipman and J. Olivié, *J. Liq. Chromatogr.*, 2 (1979) 1407.
11 B. Fransson, U. Ragnarsson and Ö. Zetterqvist, *J. Chromatogr.*, 240 (1982) 165.
12 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
13 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
14 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977, p. 301.
15 C. T. Wehr, in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. 1, CRC Press, Boca Raton, 1984, p. 31.
16 A. Tilly-Melin, Y. Askemark, K.-G. Wahlund and G. Schill, *Anal. Chem.*, 51 (1979) 976.
17 W. Jost, K. Unger and G. Schill, *Anal. Biochem.*, 119 (1982) 214.
18 J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1970.