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PERFLUOROALKANOIC ACIDS AS LIPOPHILIC ION-PAIRING REAGENTS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY OF PEPTIDES INCLUDING SECRETIN

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SUMMARY

The influence of various perfluoroalkanoic acids, as lipophilic ion-pairing reagents, on the retention and selectivity of underivatized hexa- and heptacosapeptides was studied on octadecylsilyl-silica. The retention, and in several cases the selectivity, increases in the series from trifluoroacetic acid to perfluorodecanoic acid. With perfluorooctanoic and perfluorodecanoic acid it was possible for the first time to follow directly the aspartyl-glycyl($\alpha \rightarrow \beta$)-rearrangement in the gastrointestinal 27-peptide hormone secretin.

INTRODUCTION

High-performance liquid chromatography (HPLC) is used to an increasing extent in peptide chemistry. It can be employed to follow reactions, in the purification of products and to check on purity, especially by the application of reversed-phase chromatography and of ion-pairing reagents[1–3].

The analysis and purification of unprotected peptides can be effected by adding trifluoroacetic acid to the eluent[4,5]. However, the selectivity between closely related unprotected peptides on octadecylsilyl-silica was less than with the corresponding protected peptides[6].

The addition of alkyl sulphonates and alkyl sulphates to the eluent has been described for the separation of many basic compounds[7–9]. With sulph(on)ates UV detection is not possible below 230 nm. Horváth *et al.*[9] have described the use of perfluorooctanoic acid and perfluorodecanoic acid for the separation of catecholamines, but they did not obtain reproducible results.

We have investigated the change of selectivity between unprotected peptides as a function of the lipophilicity of the ion-pairing reagent.

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TABLE I

THE k' VALUES OF SECRETIN AND ANALOGUES

$k' = (V_{peptide} - V_{water})/V_{water}$. Standard abbreviations are used for amino acids[14].

*Ion-pairing reagent**	*n*											
	0		*1*		*2*		*5*		*6*		*8*	
	5 mM	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*
Secretin (S)	1.3	2.2	2.7	5.7	8.5	19.0	1.9	5.4	5.8	15.6	4.8	12.5
[Aspartoyl[2]]-S	1.2	2.2	2.8	5.7	9.2	19.9	2.2	6.4	6.9	18.6	5.9	15.1
[β-Asp[3]]-S	1.3	2.2	2.7	5.2	7.9	16.5	1.7	4.7	5.0	13.4	4.0	10.7
[Ala[1]]-S	1.6	2.9	2.7	5.8	7.7	15.7	1.2	3.0	3.0	6.9	2.0	5.0
[desamino-His[1]]-S	1.6	2.8	2.9	6.0	8.0	15.6	1.2	3.0	3.0	7.1	2.0	4.2
Eluent, methanol–water		65:35		67:33		67:33		79:21		79:21		85:15

* Perfluoroalkanoic acids, $CF_3(CF_2)_nCOOH$, were used.

TABLE II

THE k' VALUES OF HEXAPEPTIDES

Details and footnote as in Table I.

Ion-pairing reagent	*n*											
	0		*1*		*2*		*5*		*6*		*8*	
	5 mM	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 Mm*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*
S(1–6)-NH_2	4.0	5.5	6.4	8.9	3.0	3.7	3.2	4.6	3.2	4.3	5.8	9.5
[Aspartoyl[2]]-S(1–6)-NH_2	3.9	5.7	6.6	9.2	3.2	3.9	3.5	4.7	3.3	4.6	6.0	9.6
[β-Asp[3]]-S(1–6)-NH_2	3.4	4.8	5.3	7.7	2.7	3.4	3.0	4.1	3.0	4.0	5.4	8.5
S(13–18)	1.3	2.2	3.9	7.2	3.6	5.0	8.4	14.5	9.0	15.3	25.7	32.2
[β-Asp[15]]-S(13–18)	0.9	1.5	2.9	5.5	3.1	4.5	7.9	13.9	8.5	14.9	24.6	32.1
Eluent, methanol–water		13:87		20:80		40:60		60:40		65:35		70:30

EXPERIMENTAL

Chemicals and materials

All peptides were synthesized in this laboratory. Amino acid residues are of the L configuration. The synthetic segments of porcine secretin are designated S(n–m), in which n and m are the sequence numbers of the corresponding amino acid residues in secretin, if the histidine residue occupies position 1. The following peptides were used: porcine secretin (S) (Fig. 1), [desamino-His[1]]-S, [aspartoyl[3]]-S, [Ala[1]]-S, [β-Asp[3]]-S, S(1–6)-NH_2, [aspartoyl[3]]-S(1–6)-NH_2, [β-Asp[3]]-S(1–6)-NH_2, S(13–18), [β-Asp[15]]-S(13–18).

H - His - Ser - Asp - Gly - Thr - Phe - Thr - Ser - Glu - Leu - Ser - Arg - Leu - Arg -
1 2 3 4 5 6 7 8 9 10 11 12 13 14

- Asp - Ser - Ala - Arg - Leu - Gln - Arg - Leu - Leu - Gln - Gly - Leu - Val - NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27

Fig. 1. The sequence of porcine secretin.

Reagent-grade methanol was used and mixed on a volume-to-volume basis with freshly distilled water. The ion-pairing reagents used were: perfluorodecanoic acid (PCR Research Chemicals, Gainesville, FL, U.S.A.), perfluorooctanoic acid and perfluoroheptanoic acid (Riedel-de Haen, Hannover, G.F.R.), heptafluorobutyric acid and trifluoroacetic acid (Aldrich, Milwaukee, WI, U.S.A.) and pentafluoropropionic acid (E. Merck, Darmstadt, G.F.R.).

Apparatus

A Waters Model 6000 A pump with a Model U6K injector was used in combination with a Pye Unicam LC 3 or a Schoeffel Spectroflow Monitor SF 770 for variable-wavelength UV detection. The column (15 × 0.4 cm I.D.) was packed[10] with Nucleosil C_{18}, 7 μm (Macherey, Nagel & Co., Düren, G.F.R.).

Procedures

The peptides were dissolved in the eluent (1 mg/ml). The eluents were degassed by careful filtration under reduced pressure, after which the alkanoic acid was added. The observed pH of the eluents varied between 2.1 and 2.5. The column was kept at ambient temperature. After use it was flushed, via a gradient, with methanol and stored in this solvent. The wavelength of the UV detection was set between 205 and 225 nm, depending on the alkanoic acid, its concentration and the UV detector.

RESULTS AND DISCUSSION

The use of perfluoroalkanoic acids in the present concentration range makes UV detection possible at 200 nm up to 220 nm, depending on the acid used, since peptides absorb fairly strongly at these wavelengths.

The influence of the perfluoroalkanoic acids on the capacity factors of secretin, partial sequences and their analogues is summarized in Tables I and II. The retention greatly increases with increasing chain length of the counter-ion, so that the percentage of methanol in the eluent in the series of trifluoroacetic acid to perfluorodecanoic acid

had to be raised in order to get usable retention times. Increase of the concentration of the counter-ion causes an increase of the retention. The influence of the chain length of the counter-ion on the retention is much greater with the 6- than with the 27-peptides.

The separation between secretin and its analogues was improved by increasing the chain length of the ion-pairing reagent (Fig. 2). The change of the methanol percentage is probably not the primary cause for the selectivity change, because with pentafluoropropionic acid and heptafluorobutyric acid a change of selectivity occurred in the same solvent system (Table I, Fig. 2). Both [Ala^1]-S and [desamino-His^1]-S have one basic group less than secretin, which accounts for their retention becoming less than that of secretin when the chain length of the counter-ion increases (Table I, Fig. 2). The same behaviour is found with S(13–18) and S(1–6)-NH_2, containing one basic group less (Table II, Fig. 3). The concentration of the counter-ion has little influence on the selectivity.

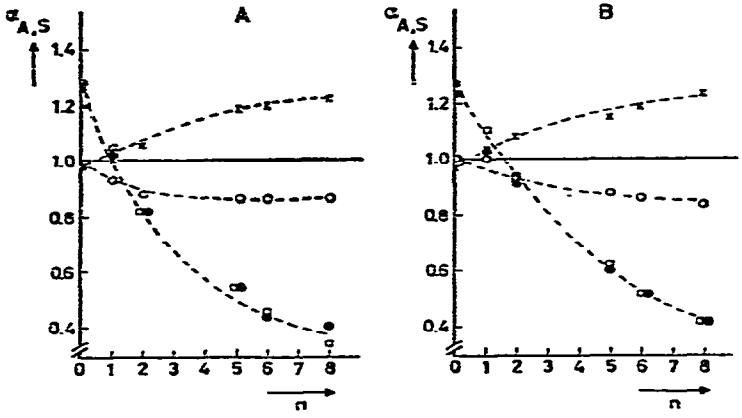

Fig. 2. The selectivity, $\alpha_{A,S} = k'$ (analogue)/k' (secretin), between secretin and some analogues with different counter-ions, $CF_3(CF_2)_nCOOH$, on a Nucleosil C_{18} column. Eluent: methanol–water (see Table I). Concentration of counter-ion: 0.01 *M* (A) and 0.005 *M* (B). ×, [Aspartoyl^3]-S; ○, [β-Asp^3]-S; ●, [Ala^1]-S; □, [desamino-His^1]-S.

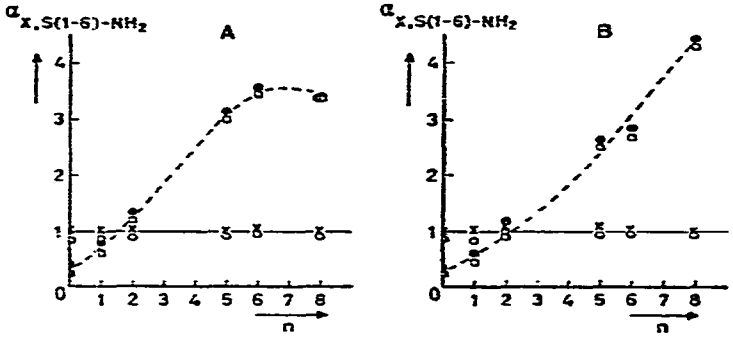

Fig. 3. The selectivity, $\alpha_{A,S(1-6)-NH_2} = k'$ (analogue)/k' (S(1–6)-NH_2), between S(1–6)-NH_2 and some hexapeptides with different counter-ions, $CF_3(CF_2)_nCOOH$, on a Nucleosil C_{18} column. Eluent: methanol–water (see Table II). Concentration of counter-ion: 0.01 *M* (A) and 0.005 *M* (B). ●, S(13–18); □, [β-Asp^{15}]-S(13–18); ×, [aspartoyl^3]-S(1–6)-NH_2; ○, [β-Asp^3]-S(1–6)-NH_2.

Two mechanisms account for the effects of the addition of lipophilic ions to the eluent[11]. In the case of "dynamic ion exchange" the lipophilic ion is strongly adsorbed on the stationary phase (*e.g.*, octadecylsilyl-silica), which takes on the properties of an ion exchanger[12,13]. Thus, separation is mainly based on the number and strength of the ionic groups of the solute. With "ion-pair chromatography" the ionized solute and a lipophilic counter-ion form a neutral pair in the eluent, which can be adsorbed on the stationary phase[9]. The number of basic groups and the lipophilicity of the solute and the counter-ion determine the retention. In order to get an impression of the mechanism, the capacity factors of the perfluoroalkanoic acids were determined in the solvent systems used for the separations, but without the acid. For the eluents used with the heptacosapeptides (Table I), the capacity factors (k') varied from 0.2 (trifluoroacetic acid) to 0.6 (perfluorodecanoic acid). The eluents used with the hexapeptides (Table II) gave higher values, 0.2 (trifluoroacetic acid) to 2.1 (perfluorodecanoic acid). This indicates that the adsorption of the perfluoroalkanoic acids on octadecylsilyl-silica is low for the eluents used for the heptacosapeptides and low to moderate for the eluents used for the hexapeptides.

We conclude that mainly ion-pair formation in the eluent takes place. However, with perfluoroheptanoic acid, perfluorooctanoic acid and perfluorodecanoic acid in the eluents used for the hexapeptides a dynamic ion-exchange mechanism cannot be excluded. The increase of the selectivity for the separation of secretin, [aspartoyl^3]-S and [β-Asp^3]-S can be best explained by the formation of an ion-pair, resulting in shielding of the hydrophilic parts of the molecule. Changes in the con-

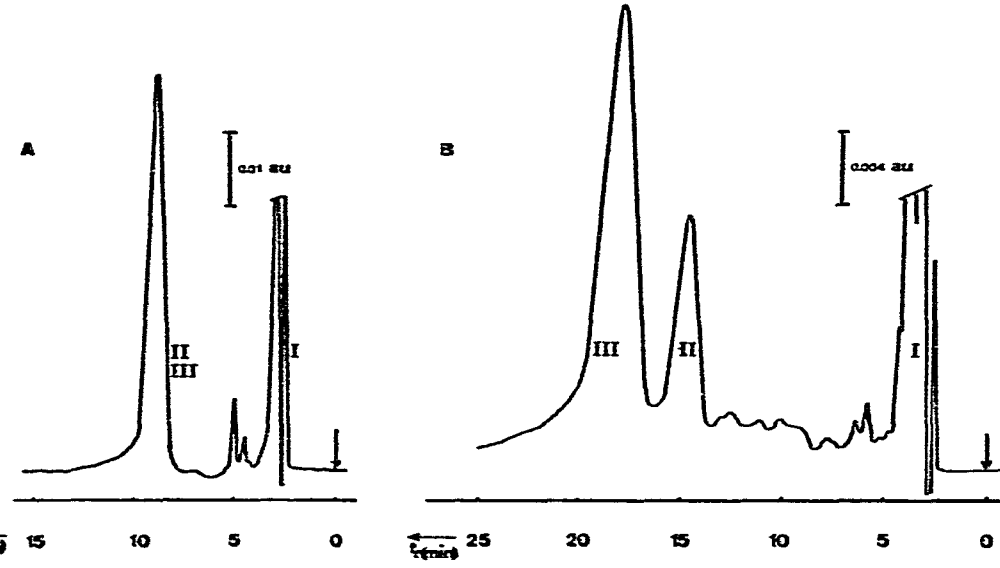

Fig. 4. The separation of products formed by rearrangement of synthetic porcine secretin. Secretin was dissolved (c = 0.5 mg/ml) in 0.1 *M* ammonium acetate (adjusted to pH 7.5). After storage at 50°C during 65 h, 10 μl (A) or 40 μl (B) were injected. Column: Polygosil C_{18} (10 μm), 30 × 0.4 cm I.D. Eluents: A, methanol–water–trifluoroacetic acid (65:35:0.1); B, methanol–water (82:18) with 0.005 *M* perfluorooctanoic acid. UV detection: A, 205 nm; B, 215 nm. Flow-rate: 1 ml/min. Peaks: I = solvent; II = [β-Asp^3]-secretin; III = secretin.

formation and/or configuration of the hydrophobic part of the molecule can thus influence the retention, as in protected peptides, where relative small changes affect the retention.

Our research on a series of perfluoroalkanoic acids has appreciably extended the analysis of underivatized peptides. The gastrointestinal 27-peptide hormone secretin (Fig. 1) and some closely related derivatives can now be separated.

It appeared to be possible to follow directly the aspartyl-glycyl($\alpha \rightarrow \beta$)-rearrangement in secretin with perfluorooctanoic acid or perfluorodecanoic acid in the eluent (Fig. 4). Details of this rearrangement will be published elsewhere.

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