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THIN-LAYER CHROMATOGRAPHY OF CLOSELY RELATED POLYPEPTIDES ON SILANIZED SILICA GEL

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SUMMARY

The chromatographic characteristics of 13 polypeptides were studied on home-made layers of silanized silica gel alone and impregnated with N-dodecylpyridinium chloride and also on RP-2 plates. The influence of the type and percentage of organic solvent, of the pH and ionic strength of the eluent and of the percentage of detergent on the layer was investigated. Attempts were made to understand the retention mechanism of the polypeptides in relation to their molecular weight, charge and structure. Hydrophobic and electrostatic interactions are responsible for the chromatographic behaviour of the different compounds. Separations of five actinomycins, of angiotensins and of other polypeptides were carried out on RP-2 plates and on layers of silanized silica gel impregnated with N-dodecylpyridinium chloride.

INTRODUCTION

In previous papers[1–3], the usefulness of layers of silanized silica gel (C_2) alone and impregnated with anionic or cationic detergents in the separation of amino acids and peptides with molecular weights up to 750 was discussed. The literature[4] indicates that the separation of peptides of moderate size (molecular weight 700–3000) of similar structure is a difficult analytical problem of great importance.

Recently this subject has been studied using high-performance liquid chromatography (HPLC) on silanized silica gel (C_2, C_8 and C_{18}) alone[4–8] and in the presence of ion pairs[9–11]. We therefore considered it useful to employ reversed-phase thin-layer chromatography (TLC) with or without detergents for the separation of closely related peptides with molecular weights in the range 897–3495, particularly angiotensins and actinomycins, which have recently been studied by HPLC[5,12,13]. The aim of this work was also to understand the interactions of the polypeptides with the stationary phase and therefore their chromatographic behaviour. Both home-made layers of silanized silica gel and ready-for-use RP-2 plates were used.

EXPERIMENTAL

Solutions of angiotensins, melittin, glucagon and insulin-B (Serva, Heidelberg,

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TABLE I

R_F VALUES OF POLYPEPTIDES ON HOME-MADE LAYERS OF SILANIZED SILICA GEL (SSG) AND ON RP-2 PLATES

Eluents: (1) 1 *M* acetic acid in 30% methanol; (2) 1 *M* acetic acid in 50% methanol; (3) 1 *M* acetic acid + 3% potassium chloride in 50% methanol; (4) 1 *M* acetic acid + 3% potassium chloride in 60% methanol; (5) water–methanol–tetrahydrofuran (40:30:30); (6) 3% potassium chloride in water–methanol–tetrahydrofuran (40:30:30).

Compound	*SSG*			*RP-2*				*Molecular weight*	*Σhn**
	1	*2*	*3*	*3*	*4*	*5*	*6*		
Angiotensin III inhibitor	0.47	0.86	0.76	0.75	0.89	0.00	0.81	897	6.82
Angiotensin III	0.16	0.69	0.55	0.53	0.74	0.00	0.71	931	7.07
Angiotensin II	0.37	0.83	0.75	0.73	0.88	0.02	0.79	1046	7.05
Des-Asp angiotensin I	0.24	0.79	0.63	0.59	0.81	0.01	0.75	1181	8.83
Angiotensin I	0.22	0.79	0.63	0.60	0.83	0.01	0.76	1297	8.81
Melittin	0.00	0.06	0.00	0.00	0.00**	0.00	0.40**	2840	19.88
					0.03**		0.44**		
					0.06***		0.52***		
Glucagon	0.00	0.44	0.33	0.26	0.68	0.00	0.72	3483	9.96
Insulin-B	0.00	0.41	0.36	0.31	0.73	0.00	0.72	3495	–
Actinomycin C_1	0.00	0.04	0.03	0.02	0.13	0.21	0.25	1255.5	–
Actinomycin C ($C_1 + C_2 + C_3$)	0.00	0.04	0.03	0.02	0.10***	0.14***	0.19***	–	–
					0.13***	0.18***	0.22***		
						0.22**	0.25**		
Actinomycin V	0.00	0.05	0.04	0.03	0.15	0.18	0.22	1269.5	–
Actinomycin I	0.00	0.09	0.08	0.05	0.21	0.30	0.32	1271.5	–

* Summation of the "hydrophobicity numbers" of the amino acid side-chains present in the polypeptides[15,16].

** Secondary spot.

*** Main spot.

G.F.R.) and of actinomycins (Serva and Roth, Karlsruhe, G.F.R.) were prepared by dissolving about 1 mg of substance in 1 ml of water–methanol (4:6). Volumes of 1 μl of the solutions were deposited on the layers, except for melittin, glucagon and insulin-B, for which 2 μl were used. With the RP-2 plates (Merck, Darmstadt, G.F.R.) 0.2–0.5 μl of standard solutions was employed.

The spots of actinomycins were yellow; the other compounds were rendered visible with a 1% solution of ninhydrin in pyridine–acetic acid (5:1)[1]. The migration distance was 11 cm for the home-made layers and 6 cm for the RP-2 plates unless stated otherwise.

The measurements were carried out at 25°C with a Desaga thermostatic chamber.

Structure of polypeptides

Angiotensin III inhibitor: Arg–Val–Tyr–Ile–His–Pro–Ile.

Angiotensin III: Arg–Val–Tyr–Ile–His–Pro–Phe.

Angiotensin II: Asp–Arg–Val–Tyr–Ile–His–Pro–Phe.

Des-Asp angiotensin I: Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu.

Angiotensin I: Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu.

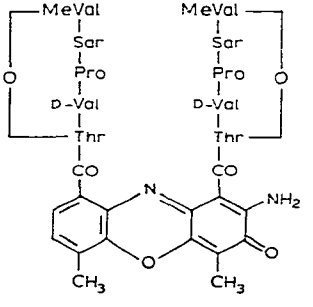
Melittin: Gly–Ile–Gly–Ala–Val–Leu–Lys–Val–Leu–Thr–Thr–Gly–Leu–Pro–Ala–Leu–Ile–Leu–Trp–Ile–Lys–Arg–Lys–Arg–Gln–Gln–NH_2. The product contains *ca.* 5% of N-formylmelittin and up to 5% of N-terminal serine analogue.

Glucagon: His–Ser–Glu–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–Asp–Ser–Arg–Arg–Ala–Glu–Asp–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr.

Insulin-B chain from bovine: Phe–Val–Asn–Gln–His–Leu–Cys–Gly–Ser–His–Glu–Ala–Leu–Tyr–Leu–Val–Cys–Gly–Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala. The two cysteine residues are oxidized to cysteic acid.

Actinomycin C composition: 8% C_1, 46% C_2, 46% C_3. Actinomycin C_2 differs from C_1 by having one of its two D-valines replaced by D-alloisoleucine; C_3 has both D-valines substituted by D-alloisoleucine. Actinomycins I and V differ from C_1 by having one of the two prolines replaced by 4-hydroxy- and 4-ketoproline, respectively.

Actinomycin C_1, D, IV[14]:



RESULTS AND DISCUSSION

Layers of silanized silica gel

Table I lists the chromatographic characteristics of the polypeptides on home-

made layers of silanized silica gel and on RP-2 plates, under different elution conditions.

Eluting with 1 *M* acetic acid in 30% methanol (column 1), only the first layers can be used owing to the non-wettability of the RP-2 plates[17–20]. With such an eluent, the actinomycins and the three polypeptides with the highest molecular weights remain at the point of application whereas the angiotensins are less retained, owing to protonation of the amino groups of their basic amino acid residues and, except for angiotensin I and des-Asp angiotensin I, migrate in different ways. The separation of angiotensin I from angiotensin II is very important for the detection of renin activity, as these two polypeptides are formed from the renin substrate by renin and converting enzyme[5]. The affinity sequence of angiotensin I and II is similar to that found in acidic medium on C_{18} columns, and has been ascribed to their different sizes and to the presence in the angiotensin I of the C-terminal His–Leu with hydrophobic characteristics[5].

The affinity sequence of the five angiotensins, however, is not completely in agreement with the order of their molecular weights. The greatest anomaly is shown by the behaviour of angiotensin III, which, notwithstanding its low molecular weight, is the more strongly retained. The influence of the polarity of the polypeptides, that is, of the parameter that is currently taken into account in order to explain the chromatographic behaviour of these compounds, is evident from the higher retention of angiotensin III with respect to angiotensin III inhibitor. In fact, as shown by the total "hydrophobicity numbers" (Σhn) of the different amino acid side-chains present in the various peptides (see Table I), the first compound has more hydrophobic characteristics than the second, in accordance with the presence in its molecule of the C-terminal phenylalanine, which is more hydrophobic than the C-terminal isoleucine in the angiotensin III inhibitor. It is well known that the affinity of a polypeptide towards silanized silica gel is greater the more hydrophobic the constituent amino acids are[4,21]. In this respect the C-terminal amino acid of the peptides seems to be of particular importance[2,3].

Such a parameter, however, does not explain the behaviour of the five angiotensins because, for instance, the presence of the initial residue of aspartic acid does not involve a substantial change in the Σhn values of angiotensin III with respect to angiotensin II and of des-Asp angiotensin I with respect to angiotensin I, but it causes a marked difference in the R_F values of the first two compounds, greater than that which could be foreseen. Further, angiotensin III is the most retained among the five compounds notwithstanding the order of the Σhn values.

It follows, therefore, that if ionogenic groups are present, the retention of polypeptides cannot be explained on the basis solely of the hydrophobicity as it also depends on the polypeptide charge and on the position of charged sites in the side-chain, other than on the conformation in solution of the macromolecule.

Influence of the organic solvent concentration. As the percentage of organic solvent in the eluent is increased (see column 2 in Table I), the hydrophobic interactions are reduced and a general increase in the R_F values for all the compounds with a levelling in the retention of the angiotensins is observed.

With this eluent, glucagon and insulin-B could be separated from melittin and actinomycins. Bearing in mind that the first two polypeptides have the highest molecular weights, it is concluded that the size of these compounds is not the parameter

that determines their retention on layers of silanized silica gel under these elution conditions.

The smaller retention of glucagon with respect to melittin is in agreement with the sequence of their Σhn values, as the hydrophobic characteristics of the former compound are much less marked than those of the latter.

The behaviour of insulin-B, the least retained of the three polypeptides, cannot be explained on the basis of its Σhn value as it is higher than that of glucagon; its behaviour is correlated with the presence of sulphonic groups in the two cysteic acid residues (see Experimental) which involve negative charges in the macromolecule. With very high percentages of organic solvent in the eluent (70%), the angiotensins, glucagon and insulin-B migrate with the solvent front, whereas melittin (R_F = 0.67) and actinomycins (R_F 0.47–0.54) are still retained by hydrophobic interactions.

With the same eluents very elongated spots, which start from the point of application, are obtained on RP-2 layers for angiotensins and for the three polypeptides with the highest molecular weights, whereas actnomycins give compact spots whose R_F values are similar to those obtained on the home-made layers.

On RP-2 plates the elution time decreases from 100 min on eluting with 1 *M* acetic acid in 50% methanol to 40 min in the presence of 70% methanol.

Influence of ionic strength and pH of the eluent. Columns 3 and 4 in Table I list the R_F values of the compounds on home-made and RP-2 layers on eluting with 1 *M* acetic acid in 50% methanol containing 3% of potassium chloride. Comparison of the results in column 3 with those in column 2 shows that an increase in the ionic strength of the mobile phase increases the retention of the polypeptides. Such behaviour can be ascribed to the increase in the hydrophobic interactions between these compounds and the stationary phase[22,23] rather than to the salting-out effect[24].

On RP-2 plates the same occurrence is observed but elongated spots are no longer obtained and, therefore, under such elution conditions the use of these plates is very suitable for analytical purposes. On these layers the presence of potassium chloride in the eluent plays a double role in increasing the hydrophobic interactions and in reducing or eliminating the adsorption correlated with hydrogen bonding and/or with coulombic interactions[22], which are responsible for the elongated spots observed in the absence of the salt.

As the methanol concentration in the eluent is increased, the spots remain compact on both layers and a general decrease in the retention is observed for all of the compounds owing to reduction of the hydrophobic interactions. Column 5 in Table I gives the R_F values on RP-2 plates relative to 1 *M* acetic acid + 3% potassium chloride in 60% methanol as eluent. It should be noted that under the conditions used the mixture of the three actinomycins of the C group gives two spots and, surprisingly, melittin gives three spots, of which the main spot has the highest R_F. The two secondary spots can be ascribed to the presence of by-products in the commercial compound (see Experimental). Salt solutions containing higher percentages of methanol (70%) cannot be used since in this medium precipitation of the salt occurs.

On eluting with water–methanol–tetrahydrofuran (4:3:3) (column 6), the angiotensins and the three polypeptides with the highest molecular weights remain at the point of application, whereas actinomycins migrate to different extents and the three of the C group are well separated.

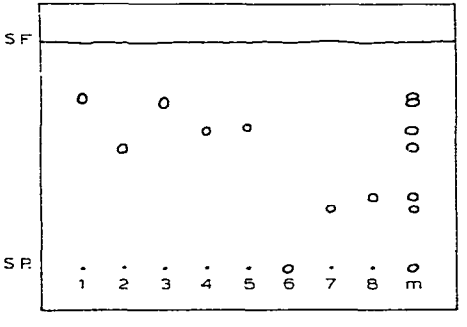
The greater retention of the polypeptides with this eluent than with the eluent

containing acetic acid can be ascribed to the higher pH of the mobile phase and to the stronger ionic interactions between polypeptides and the free silanol groups at the surface of the stationary phase[25]. Under such conditions the polypeptides (except insulin-B) are not in the protonated form but mainly in the zwitterionic form, which is more strongly retained by the silanized silica gel, as was observed with amino acids[1] and small peptides[2,3].

The role of the silanol groups in the retention of peptides is increased by the absence from the eluent of an electrolyte, even if not much dissociated, such as acetic acid. The actinomycins, in contrast, the retention of which is mainly dependent on hydrophobic interactions, migrate on eluting with water–methanol–tetrahydrofuran; their R_F values, however, are smaller than those obtained with an analogous mixture containing 1 *M* acetic acid. The separation of the actinomycins of the C group must be ascribed to the presence in the eluent of tetrahydrofuran which, being less polar than methanol, reduces the affinity of these compounds towards the stationary phase, whereas it increases the differences in their polarity and therefore in the retention. The R_F sequence ($C_1 > C_2 > C_3$) in fact follows that of their polarity[13], as with actinomycins I, C_1 and V ($I > C_1 > V$) (see Experimental). This last sequence is different from that observed in absence of tetrahydrofuran ($I > V > C_1$) and this occurrence is important for analytical purposes, as it allows the simultaneous separation of the five actinomycins.

In the presence of 3% of potassium chloride (column 7), a considerable increase in the R_F values of angiotensins and of the other three polypeptides together with a small increase for actinomycins is observed. Owing to the presence of potassium chloride in the eluent, as already pointed out, the ionic interactions between the compounds and the stationary phase are drastically reduced.

Analytical applications. On the basis of the chromatographic characteristics of the polypeptides under the elution conditions reported in Table I, many separations could be effected; we carried out those shown in Figs. 1 and 2.



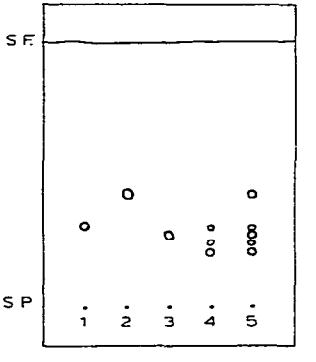


Fig. 1. HPTLC of polypeptides on RP-2 plates. Eluent, 1 *M* acetic acid + 3% potassium chloride in 50% methanol; development time, 100 min. 1 = Angiotensin III inhibitor; 2 = angiotensin III; 3 = angiotensin II; 4 = des-Asp angiotensin I; 5 = angiotensin I; 6 = melittin; 7 = glucagon; 8 = insulin-B; m = mixture of 1–8. S.P. = Start point; S.F. = solvent front.

Fig. 2. HPTLC of actinomycins on RP-2 plates. Two successive developments with 1 *M* acetic acid + 3% potassium chloride in 60% methanol. Migration distance, 7 cm. 1 = Actinomycin C_1; 2 = actinomycin I; 3 = actinomycin V; 4 = actinomycin C ($C_1 + C_2 + C_3$); 5 = mixture of 1–4. S.P. = Start point; S.F. = solvent front.

In the first instance we tried to separate the five angiotensins and the other three polypeptides on RP-2 plates by eluting with 1 *M* acetic acid + 3% potassium chloride in 50% methanol. It should be noted that the separation of all the eight compounds is not complete, as angiotensin I and des-Asp angiotensin I cannot be separated and angiotensin II and angiotensin III inhibitor give rise to two incompletely resolved spots.

As regards the angiotensins, the best results (four well separated compounds) are achieved on home-made layers by eluting with 1 *M* acetic acid in 30% methanol. With two successive developments in the same eluent the separations shown in Fig. 2 are obtained; these separations include the actinomycins of the C group and all five actinomycins on RP-2 plates.

The first separation can be effected on the same layers also by eluting with 4:3:3 and 4:2:4 water–methanol–tetrahydrofuran mixtures alone and in the presence of 1 *M* acetic acid or 3% of potassium chloride.

Layers of silanized silica gel impregnated with N-DPC

Table II lists the R_F values of the polypeptides on layers of silanized silica gel impregnated with 1% and 4% N-DPC (columns 1 and 2) on eluting with 1 *M* acetic acid in 30% methanol. Comparison with the data obtained in the absence of detergent (see column 1 in Table I) shows that the presence of the detergent on the layer reduces the retention of the polypeptides. Such a reduction becomes more marked as the detergent concentration increases. This behaviour must be ascribed to the repulsive interactions between the positive charges of the polypeptides and the functional group of the detergent adsorbed on the layer.

TABLE II

R_F VALUES OF POLYPEPTIDES ON HOME-MADE LAYERS OF SILANIZED SILICA GEL IMPREGNATED WITH 1% AND 4% N-DPC

Eluents: (1) 1 *M* acetic acid in 30% methanol; (2) 0.1 *M* acetic acid + 0.1 *M* potassium chloride in 30% methanol (pH 3.30); (3) water; (4) water–acetic acid (99.5:0.5).

Compound	*SiO_2 + 1% N-DPC: eluent 1*	*SiO_2 + 4% N-DPC*			
		1	*2*	*3*	*4*
Angiotensin III inhibitor	0.83	0.86	0.83	0.85	0.79
Angiotensin III	0.69	0.82	0.65	0.84	0.63
Angiotensin II	0.71	0.84	0.55	0.33	0.33
Des-Asp angiotensin I	0.71	0.84	0.60	0.81	0.58
Angiotensin I	0.69	0.82	0.51	0.51	0.40
Melittin	0.00	0.76	0.00	0.00	0.00
Glucagon	0.00	0.72	0.00	0.00	0.00
Insulin-B	0.00	e.s.*	0.00	0.00	0.00

* Elongated spot.

With polypeptides containing acidic amino acid residues, such as glucagon and insulin-B, the simultaneous presence of an anion-exchange process together with the usual hydrophobic interactions cannot be excluded. Insulin-B, for instance, is the most retained and this behaviour is related to the presence in its molecule of cysteic

acid residues. On eluting with 1 *M* acetic acid in 20% methanol, insulin-B remain at the point of application and could be separated from all the other compounds.

The actinomycins do not migrate from the point of application under the elution conditions in Table II, owing to their marked hydrophobic characteristics and, therefore, their R_F values are not reported.

An increase in the ionic strength of the eluent on addition of potassium chloride causes, as expected, an increase in retention. This increase is small for the angiotensins but very high for the other polypeptides and seems to be related to the reduction of the repulsive interactions between these compounds and the detergent on the layer.

An increase in the apparent pH of the eluent, at a given ionic strength and methanol concentration, results in a considerable decrease in the R_F values (column 3) and a different order of retention of the angiotensins. As an example, angiotensin III, which on layers of silanized silica gel alone is always the most strongly retained, exhibits an affinity consistent with that expected from the size of the five angiotensins. Also under these conditions, however, there is not full agreement between the affinity sequence of peptides and the order of their molecular weights or Σhm values.

The decrease in the R_F values of the polypeptides, which becomes greater as the apparent pH increases, can be ascribed to the occurrence of an anion-exchange process. On eluting with 0.1 *M* sodium acetate in 30% methanol all the compounds remain at the point of application. The impregnated layers can be used also with an eluent containing water concentrations higher than 70%[1–3] and this characteristic is very important from an analytical standpoint. Elution with water or with aqueous solutions containing small percentages of acetic acid is possible (see columns 4 and 5) and causes dramatic changes in the order of retention of the five angiotensins.

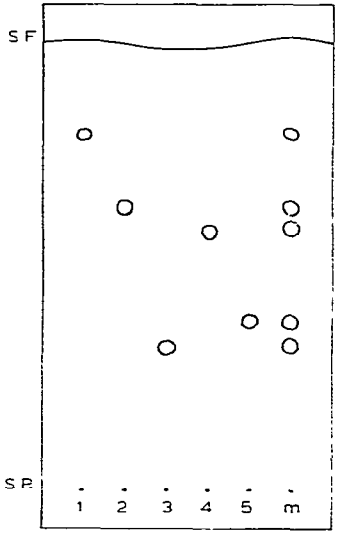


Fig. 3. Thin-layer chromatogram of angiotensins on silanized silica gel impregnated with 4% N-DPC. Eluent, water–acetic acid (99.5:0.5); migration distance, 12 cm. 1 = Angiotensin III inhibitor; 2 = angiotensin III; 3 = angiotensin II; 4 = des-Asp angiotensin I; 5 = angiotensin I; m = mixture of 1–5. S.P. = Start point; S.F. = solvent front.

The stronger retention of angiotensin II and angiotensin I with respect to angiotensin III and des-Asp angiotensin I, respectively, on eluting with water, should be noted. Such differences, which decrease with the increase in the acetic acid content in the eluent, must be ascribed to the presence of the initial residue of aspartic acid in the first two angiotensins, which interacts through an anion-exchange process with the detergent on the layer.

Fig. 3 shows the separation of the five angiotensins on impregnated layers with water–acetic acid (99.5:0.5) as the eluent. The layers of RP-2 impregnated with N-DPC can be used only with eluents containing methanol concentrations higher than 40% and, therefore, do not have any advantage in the separation of angiotensins in comparison with non-impregnated plates.

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